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UNIVERSITY OF ALBERTA

EFFECTS OF SULFIDE ON RAT HIPPOCAMPAL NEURONS

BY

© ROBERT J. BALDELLI

A THESIS SUBMITTED TO THE FACULTY OF
GRADUATE STUDIES AND RESEARCH IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1990

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

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TO MY PARENTS

ABSTRACT

The industrial toxin, hydrogen sulfide (H_2S), has a variety of neurological effects including retrograde amnesia, and acute toxicity results from failure of central respiratory drive. However, the mechanism of H_2S toxicity is poorly understood. Using the rat hippocampus *in vivo* and *in vitro*, we have studied the mechanism(s) of H_2S action on CA1 pyramidal neurons.

Results from extracellular recordings of single CA1 pyramidal cells *in vivo* suggested that HS^- reversibly inhibits spontaneously active CA1 cells. These observations are supported by results obtained from both intra- and extracellular recordings from area CA1 using the rat hippocampal slice, *in vitro*.

Using standard *in vitro* techniques, I have been able to demonstrate that toxicologically relevant concentrations of HS^- reversibly suppress synaptic transmission and cause a direct membrane hyperpolarization. The results suggest that this initial HS^--mediated hyperpolarization seen in CA1 cells is most likely due to an increased K^+-conductance. While the nature of the K^+-conductance(s) affected is still unknown, evidence suggests a Ca^{2+}-dependent K^+-conductance is involved. Furthermore, HS^- clearly causes a decrease in input resistance of the soma, and thus possibly also of the dendrites or the dendritic spines of CA1 pyramidal cells. As well, HS^- may cause a reduction in the release of excitatory neurotransmitters from presynaptic terminals which synapse onto the dendrites of CA1 neurons.

After higher concentrations of HS^- ($> LD_{90}$) are applied to intracellularly

recorded CA1 cells *in vitro*, a further hyperpolarization is observed subsequent to washout. This HS^--mediated "washout hyperpolarization" is most likely due to the reactivation of the Na^+-K^+ ATPase as it is not blocked by any of the K^+-channel blockers (except Cs^+), but is eliminated by the Na^+-K^+ ATPase inhibitor, strophanthidin.

These results suggest that inhibition of neuronal activity by H_2S (e.g. retrograde amnesia) may involve supression of synaptic response and direct hyperpolarization. Should similar actions of HS^- occur in the brainstem, they may account for the fatal loss of central respiratory drive seen in acute intoxication.

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TABLE OF CONTENTS

CHAPTER PAGE

I. INTRODUCTION 1

A. Background 2

B. Potential Sources of Exposure 3

C. Terminology 3

D. Absorption, Distribution, and Elimination of H_2S 6

E. Effects of H_2S Reported in Humans and Animals 7

F. Studies Investigating the Possible Mechanism(s) of Toxicity Due to Exposure of High Concentrations of H_2S 12

G. Statement of Purpose 15

II. MATERIALS AND METHODS 17

I. *In Vivo* Experiments 18

A. Experimental Animals 18

B. Anesthesia 18

C. Surgical Procedures 19

D. Stereotaxic Methods 19

E. Recording 20

1. Electrode preparation 20

2. Equipment 22

F. Physiological Identification of Cells 24

G. Drugs and Solutions 24

TABLE OF CONTENTS

CHAPTER PAGE

H. Histology 25

1. Method for recording site marking 25

2. Fixation 25

II. *In Vitro* Experiments 27

A. Preparation and Dissection 27

B. Apparatus 27

C. Electrophysiological Recording 31

1. Extracellular recording 34

2. Intracellular recording 35

3. Iontophoresis experiments 38

D. Preparation of Artificial Cerebral Spinal Fluid 40

E. Preparation of NaHS and Other Reagents 40

F. Construction of Concentration-Response Curves for NaHS 41

G. Data Analysis 42

1. Measurements of changes in membrane potential 42

2. Measurement of neuronal input resistance 43

3. Measurement of PS and EPSP amplitudes 43

4. Statistics 44

III. RESULTS 45

I. *In Vivo* Experiments 46

A. Effects of Acetylcholine and Glutamate 46

TABLE OF CONTENTS

CHAPTER PAGE

B. Effects of Iontophoretically Applied NaHS 47

C. Effects of Sulfur Containing Amino Acids 49

D. Discussion 52

II. *In Vitro* Experiments 56

A. Effects of NaHS on Membrane Properties and Responses 56

1. Initial hyperpolarization and changes in input resistance 56

2. "Washout hyperpolarization" 62

3. Effects of NaHS on synaptic transmission 62

4. Effects of NaHS on active membrane properties 66

B. Mechanism(s) of Action of NaHS 69

1. Experiments to determine the nature of the ionic conductance altered by HS^- 69

2. Experiments to determine the nature of the HS^--induced K^+-conductance 76

3. Experiments to determine the mechanism(s) involved in HS^--induced washout hyperpolarization 90

4. Effects of NaHS on synaptic transmission 96

a. Blockade of evoked synaptic transmission 96

b. Iontophoretic applications of glutamate 97

TABLE OF CONTENTS

CHAPTER PAGE

IV. DISCUSSION 101

- A. Mechanism of Hyperpolarization and Input Resistance Decrease 103
  - 1. Nature of ionic conductance altered by HS^- 103
  - 2. Nature of HS^--activated K^+-conductance 105
    - a. Voltage dependent K^+-channels 105
    - b. Inwardly rectifying K^+-conductances 106
    - c. ATP-sensitive K^+-channels 107
    - d. Leak conductances 108
    - e. Ca^{2+}-sensitive K^+-conductance 109
  - 3. HS^--mediated depolarizations observed during some experimental conditions 110
  - 4. Similarities between HS^-- and anoxic-induced effects 113
- B. Washout Hyperpolarization 115
- C. Effects on Synaptic Transmission 118
- D. Suggestions For Further Experiments 120
- E. Summary and Conclusions 122

V. REFERENCES 125

LIST OF FIGURES

FIGURE PAGE

1. Diagram of the experimental arrangement used for extracellular recording and drug iontophoresis 23
2. The "Scottish chamber" used for electrophysiological recording of area CA1 of rat hippocampal slice 30
3. A schematic diagram of a hippocampal slice showing the arrangement of the electrodes used for intra- and extracellular recording *in vitro* 33
4. Diagram of the experimental arrangement used for extracellular and intracellular recording *in vitro* 39
5. Effects of HS^-, TAU, and both together on firing rate of hippocampal CA1 neuron *in vivo* 48
6. Hippocampal CA1 neuron firing and effect of HS^-, TAU, and both together on responses to GLU pulses 50
7. Effect of different concentrations of HS^- on membrane potential, EPSP and orthodromically-evoked PS amplitudes in CA1 pyramidal neurons 57
8. Concentration-response relationships for HS^- actions on CA1 neuron properties 60
9. V-I relationship, obtained in current clamp, of CA1 neurons 61

LIST OF FIGURES

FIGURE PAGE

10. Concentration-response relationships for HS$^-$ actions on the orthodromically-evoked EPSP and PS amplitudes in area CA1 64

11. Effects of HS$^-$ on the stimulus-response relationships of the intracellularly recorded EPSP, and the extracellularly recorded PS in area CA1 of rat hippocampus 65

12. Relationship between the amplitudes of the PS and fEPSP recorded extracellularly in area CA1 67

13. Effects of HS$^-$ on action potentials of a CA1 pyramidal cell 68

14. HS$^-$-induced hyperpolarization and washout hyperpolarization responses: effects of elevation of extracellular K$^+$ concentration 71

15. I-V relationships of a CA1 pyramidal cell obtained by slow ramp depolarization during voltage clamp 74

16. HS$^-$-induced effects are unaffected by extracellular TTX and 4-AP 78

17. Application of TTX and TEA reduced the membrane hyperpolarization and decreased input resistance caused by HS$^-$ 80

18. Effects of extracellular Cs$^+$ on the effects caused by HS$^-$ 82

19. Effects of intracellular Cs$^+$ on the HS$^-$-mediated changes in membrane potential in CA1 neurons *in vitro* 84

LIST OF FIGURES

FIGURE PAGE

20. Effects of intracellular Cs^+ on the effects caused by HS^- in a CA1 cell 86

21. Intracellular injections of Mg-ATP do not effect the HS^--mediated hyperpolarization and decreased input resistance seen in CA1 pyramidal cells 87

22. Effects of intracellular injections of BAPTA on the HS^--mediated effects observed in CA1 neurons *in vitro* 89

23. Summary of the effects of K^+-channel blockers, TTX, and intracellular injections of Mg-ATP or BAPTA on the initial hyperpolarization, and decreased input resistance caused by HS^- (200 uM) on CA1 pyramidal cells *in vitro* 91

24. Summary of the effects of K^+-channel blockers, TTX, and intracellular injections of Mg-ATP or BAPTA on the HS^--induced washout hyperpolarization caused by HS^- (200 μM) on CA1 cells *in vitro* 93

25. Effects of strophanthidin on HS^--mediated washout hyperpolarization . 95

26. Effects of low Ca^{2+}, high Mg^{2+} on membrane hyperpolarization and change in input resistance caused by HS^- 98

27. HS^--mediated decrease of the amplitude of the intracellularly recorded EPSP was greater than the decrease of the responses to glutamate pulses applied iontophoretically to the dendrites of a CA1 pyramidal neuron 100

LIST OF PLATES

PLATE PAGE

1. Experimental appparatus used for electrophysiological recording of area CA1 of rat hippocampal slice 28

2. Experimental arrangement of electrodes used for electrophysiological recording of area CA1 *in vitro* 32

LIST OF ABBREVIATIONS

ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarization
ALA-S	delta-aminolaevulinic acid synthase
4-AP	4-aminopyridine
ATP	adenosine 5'-triphosphate
BAPTA	1-,2 bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid
CNS	central nervous system
CsAc	cesium acetate
CsCl	cesium chloride
E_K	potassium equilibrium potential
EPSP	excitatory postsynaptic potential
fEPSP	field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
gK_{ATP}	ATP-sensitive potassium conductance
$gK_{Ca^{2+}}$	Ca^{2+}-sensitive potassium conductance
gK_{Na^+}	Na^+-sensitive potassium conductance
GLU	glutamate
Heme-S	heme synthase
Hz	Hertz
HS^-	hydrosulfide anion

LIST OF ABBREVIATIONS

H_2S	hydrogen sulfide
I_A	"A" current
I_K	delayed rectifier
I_M	"M" current
I_Q	"Q" current
ip	intraperitoneal
I-V	current-voltage
KAc	potassium acetate
LD_{50}	median lethal dose
MAO	monoamine oxidase
MΩ	megaohm
ms	millisecond
min	minute
μ	micro
nA	nanoampere
Na_2S	sodium sulfide
NaHS	sodium hydrosulfide
NRC	National Research Council
PE	polyethylene
PS	population spike
sec	second
SPS	spikes per second

LIST OF ABBREVIATIONS

TAU	taurine
TEA	tetraethylammonium
TTX	tetrodotoxin
V	volt
V-I	voltage-current

CHAPTER I

INTRODUCTION

A. Background

The effects of hydrogen sulfide (H_2S) on humans have been observed accurately for more than the last two hundred and fifty years. For example, during the eighteenth century, a large number of deaths occurred as a result of a series of accidental exposures to sewer gas in Paris. At this time, a report concerning an investigation into this problem published by M. Halle mentioned that two types of distinct poisonings were involved. One type was referred to as "mitte" in which the victims suffered from inflammation of the eyes and mucous membranes. The other type of poisoning was called "plomb", and in this case, the victim would die rapidly as a result of what was believed to be asphyxiation (Halle, 1785, cited by Mitchell and Davenport, 1924). However, it was almost forty years after the Halle report when it was realized that hydrogen sulfide was the causative agent for both of these poisoning types (Smith, 1983).

H_2S is a colorless gas, and in low concentrations, possesses an odor which resembles that of rotten eggs. The range of concentrations of H_2S and their effects have been investigated by analyzing data obtained from the workplace and from experiments involving animals. The concentration range from the determined odor threshold (0.01 parts per million) to that which is life threatening (700 to 1400 parts per million) is quite large, approximately ten thousand times (Smith,1983).

B. Potential Sources of Exposure

There are many potential sources of exposure to H_2S. For example, H_2S is found naturally in volcanic gases, mines, and when sulfur-containing organic matter decomposes with incomplete oxidation (Milby, 1962). Exposure to lethal concentrations of H_2S occurs frequently among those employed in the oil and gas industry such as those working around oil and sour gas wells or in areas where petroleum products are stored and processed. Workers in chemical plants may be exposed to H_2S formed when many sulfur-containing compounds are produced (Milby, 1962). In fact, lethal H_2S poisoning has occurred in over 70 agricultural and industrial settings since it is a by-product or starting material of many manufacturing processes (Milby, 1962; Beauchamp et al., 1984).

C. Terminology

When one reviews the literature on H_2S toxicity, terms such as "acute", "subacute", and "chronic" are encountered when discussing the clinical signs and symptoms produced as a result of exposure to the gas. "Acute" refers to those effects found when a single exposure (i.e. less than 24 hours) of high concentration of toxic agent occurs and they are of rapid onset and intense in nature. Acute toxicity in humans resulting from H_2S exposure (> 700 parts per million) produces systemic poisoning and is often correlated with clinical signs of central nervous system (CNS) toxicity including convulsions, unconsciousness, and respiratory paralysis (Milby, 1962; National Research Council (NRC), 1979). When dealing with a gas such as H_2S, it is easier to assess its acute toxicity by injecting solutions of its alkali salts such as sodium sulfide (Na_2S) or sodium hydrosulfide (NaSH) into the test animal. It has been demonstrated that the systemic effects (except for airway effects; Lopez et al., 1989) due to the application of the salts are identical to those effects seen after the gas is inhaled except that the effects due to injection of the salts occur more rapidly (Beauchamp et al., 1984; Smith, 1983). Application of the sulfide salt also leads to similar brain levels of sulfide (Reiffenstein et al., 1988; Lopez et al., 1989). Often, when discussing acute toxicity, a parameter called the LD_{50} (median lethal dose) is used. It is defined as the dose (or concentration) of an agent that would be lethal to fifty per cent of a population of animals and can be expressed in mg/kg or moles/kg of body weight (Smith, 1983). In "subacute" toxicity, symptoms such as eye and respiratory tract irritations predominate in humans after exposures of H_2S ranging in concentrations from 50 to 100 parts per million for several minutes to an hour (Milby, 1962; National Research Council, 1979). Others have reported decreases in the activities of heme-synthesizing enzymes such as delta-aminolaevulinic acid synthase (ALA-S) and heme synthase (Heme-S) in humans exposed to moderate, non-fatal concentrations of H_2S (e.g. 20-500 parts per million) for about 4.5 minutes. The range of blood sulfide concentrations observed in these patients was 30-130 μg/l (P. Jappinen, 1990; Tenhunen et al., 1983). Lastly, the effects due to "chronic" H_2S poisoning are difficult to define since they are usually subjective in nature. It is questionable whether or not such a condition exists especially since rapid detoxification of H_2S occurs in the blood (Haggard, 1921; Evans, 1967; see below) and therefore, it is unlikely that there is any cumulative effect of H_2S (Bittersohl, 1971; Haggard, 1925). However, it has been suggested that repeated exposures to low concentrations of H_2S can result in symptoms such as neurasthenic effects seen in humans (Ahlborg, 1951; Illinois Institute for Environmental Quality, 1974), alterations in brain metabolism in animals (Savolainen et al., 1980; Haider et al., 1980), and some reproductive effects in animals (Barilyak et al., 1975; Hannah et al., 1989a; 1989b; Roth, 1989). Others say that chronic H_2S toxicity may actually be due to a "series of low grade acute episodes" (Ahlborg, 1951; Milby, 1962).

It is easier to classify the type of H_2S toxicity based on the clinical signs manifested, for example, due to local irritation, systemic intoxication or both as opposed to acute, subacute or chronic. This is so with any toxic substance because the effects are dependent on both the duration and intensity of the exposure (Milby, 1962; Smith, 1983). Clanachan (1979) suggested that acute H_2S intoxication is time-dependent. He showed that the concentrations of H_2S exposure required to produce unconsciousness and death in mice were higher at shorter durations of exposure. However, experiments involving lower exposure concentrations of H_2S for longer time periods were not reported. In contrast, other studies (O'Donoghue, 1961) support the idea that H_2S toxicity is related to the concentration of exposure. In this study, the rate of increase of H_2S concentration influences toxicity, but the time-concentration relationship of exposure on the lethality of the gas was not critically examined (Clanachan, 1979). More studies are required to examine more precisely the relationship between the time and concentration of H_2S exposure on biological systems. On the other hand, it is known that, in humans, death can occur from exposure to 100 parts per million H_2S for 8-48 hours or 1000 parts per million H_2S for a few minutes (National Research Council of Canada, 1981).

D. Absorption, Distribution, and Elimination of H_2S

The major route of absorption of H_2S gas is through the lungs while little absorption occurs through intact skin (Patty, 1949; Milby, 1962; Smith, 1983; Beauchamp et al., 1984). Studies using rats, dogs and other animals have shown that the major route for the metabolism and detoxification of sulfide occurs mainly in the liver and to a smaller extent in other tissues such as the kidneys and the lungs. Sulfide may also be oxidized by oxygenated hemoglobin in the blood (Dennis and Read, 1927; Patty, 1963; Vigil, 1979). The sulfide is metabolized to polysulfides, polythionates, thiosulfates and finally sulfate. Sulfate is the main metabolite found in the urine about 6 to 12 hours after exposure (Gunina, 1957; Curtis et al., 1972; Beauchamp et al., 1984; Smith, 1983). The exact nature of this conversion is unknown although it is believed that it may be both enzymatic (sulfide oxidase, ferritin) and non-enzymatic. Hence, H_2S biotransformation occurs rapidly and only the absorption of H_2S at a rate that exceeds its rate of detoxification or oxidation would lead to effects such as systemic intoxication (Vigil, 1979). The major route of elimination is via the kidneys, while some is exhaled through the lungs in its unmetabolized form.

E. Effects of H_2S Reported in Humans and Animals

When exposure levels of H_2S in humans and animals exceed 500 to 700 parts per million, systemic intoxication predominates (Milby, 1962; National Research Council, 1979). Because of its moderate solubility and its route of administration (e.g. inhalation), H_2S is rapidly absorbed, leading to high concentrations of the unoxidized gas in the bloodstream. The systemic effects are clearly manifestations of CNS toxicity (Haggard, 1925). Clinical signs of acute systemic intoxication include sudden fatigue, dizziness, intense anxiety, convulsions, loss of olfactory function, unconsciousness, collapse, and respiratory failure followed by cardiac failure and death (Ahlborg, 1951; Mitchell and Yant, 1925). Moderately high concentrations of H_2S stimulate the respiratory system (Haggard et al., 1922) and this effect is probably mediated by the chemoreceptors of the carotid body. This results in hyperpnea and on some occasions, bradycardia. Although it has been suggested that the sulfide-induced hyperpnea is due to carotid body stimulation, respiratory arrest and asphyxia seen in sulfide intoxication are believed to be a result of the "direct paralyzing effect" of H_2S on the brainstem respiratory center (Henderson and Haggard, 1943; Milby, 1962; Beauchamp et al., 1984). It should be mentioned that inhalation of high concentrations of H_2S may lead to manifestations of some of these effects after a single breath (Milby, 1962). Nonlethal systemic intoxication usually involves rapid and complete recovery and sequelae are unlikely to occur (McCabe and Clayton, 1952; Ahlborg, 1951; Milby, 1962). However, if they do occur, they are usually due to CNS damage incurred during the time of collapse and prolonged anoxia as a result of H_2S asphyxia (Milby, 1962). Sequelae are generally neurasthenic in nature and may last anywhere from days to months (Poda, 1966; Ahlborg, 1951). Symptoms include fatigue, drowsiness, headache, lack of initiative, nausea, nervousness, irritability, anxiety and depression, poor memory, decreased libido and some otoneurological symptoms such as nystagmus and disturbance of equilibrium (Poda, 1966; Ahlborg, 1951). Significant brain damage has been found in both animals and humans exposed to at least 500 parts per million of H_2S (Report on H_2S toxicity, 1988). For example, it has been shown that exposure to H_2S for 22 minutes at 500 parts per million led to necrosis of the cerebral cortex, reduction of Purkinje cells of the cerebellar cortex, and focal gliosis in rhesus monkeys (Lund and Wieland, 1966; Beauchamp et al., 1984).

As mentioned above, in subacute intoxication (e.g. exposure to 100 to 500 parts per million H_2S), irritation of the mucous membrane of the eyes and respiratory tract predominate (Milby, 1962). Prolonged exposure to these concentrations of H_2S may cause rhinitis, pharyngitis, bronchitis, pneumonia and because of its ability to penetrate deeply into the lung, it may produce inflammation of the alveoli resulting in pulmonary edema (Ahlborg, 1951; McCabe and Clayton, 1952; Sollmann, 1948; Milby 1962). Also, some effects involving central nervous system dysfunction may occur and include headache, fatigue, irritability, insomnia, and mild depression (Milby, 1962). Studies have demonstrated that when rats are exposed to these levels of H_2S, behavioral responses such as discriminated avoidance are inhibited significantly at a rate and intensity proportional to the concentrations of H_2S applied (Higuchi, 1977).

Others have examined the effects of H_2S on structure and function of the respiratory tract. For example, Lopez (1990) reported that rats exposed to H_2S concentrations from 200 to 400 parts per million via inhalation for 4 consecutive hours had: (1) severe but reversible (within 44 hours) injury to ciliated epithelium of the nasal cavity; (2) severe, irreversible injury and necrosis of olfactory cells; (3) a moderate and transient inflammatory response in the alveolar region of the lungs; and (4) a severe but transient pulmonary edema which was characterized by a notable transudation of fluid into the bronchoalveolar space. Complete reabsorption of the fluid was observed after 44 hours. He concluded that the resultant pulmonary edema originated from the "increased opening of endothelial gaps" because H_2S did not alter the morphology of the alveolar epithelium, the basal lamina, or the blood-gas barrier (pneumocytes). Hulbert et al. (1990b) observed a decrease in baseline airway caliber of lung tissue of guinea pigs that inhaled 100 parts per million H_2S for 1 hour. They hypothesized that the effects were due to inhibition of the parasympathetic nervous system. In 70 and 10 % of rats exposed to 100 and 10 parts per million H_2S for 8 hours/day, 5 days/week, for 5 weeks, via inhalation, respectively, Hulbert et al. (1990a) reported proliferation of ciliated and basal cells of lung tissue while the numbers of non-ciliated Clara cells were sharply decreased. In contrast to results obtained from the study mentioned above (i.e. effects of H_2S exposure of guinea pigs to 100 parts per million for 1 hour), these rats showed a "bronchial hyper-reactivity" to methacholine aerosol challenges, however, the underlying mechanism(s) were not known (Hulbert, 1990).

In chronic H_2S poisonings (e.g. below 100 parts per million), effects usually require repeated exposures. They are manifestations of a neurasthenic nature and symptoms include lethargy, dizziness, loss of appetite, fatigue, headache, mental depression, irritability, poor memory, abnormal peripheral reflexes, and gastrointestinal disturbances (Ahlborg, 1951; Rubin and Arieff, 1945; Report on H_2S toxicity, 1988). However, one has to be cautious because some of these manifestations may actually be sequeiae which may be as a result of previous exposures to much higher concentrations of H_2S (Ahlborg, 1951). Also, the authors conducting the studies found that it was difficult to conclude whether the symptoms observed were actually due to the direct effects of H_2S exposure (e.g. 20 parts per million) or whether these symptoms were as a result of the "psychic strain of working in an environment with a known risk of H_2S exposure" (Ahlborg, 1951; Vigil, 1979).

Numerous animal studies have been done to examine the neurological effects associated with repeated low level exposures of H_2S. For example, Sandage (1961) observed abscesses in mouse brains exposed to 20 parts per million for 90 days. It has been reported that phospholipid levels decrease and lipid peroxidation increases in the cerebral cortex and brain stem of guinea pigs exposed to 20 parts per million H_2S for 1 hour/day for 11 days. Symptoms including fatigue, somnolence, and dizziness were also observed in these animals (Haider et al., 1980). Others have reported motor chronaxie abnormalities in rats exposed to 7 parts per million for 12 hours/day for 3 months (Duan, 1959).

Some studies have examined the effects of exposure of chronic low dose H_2S on brain development in fetuses. For example, Hannah et al. (1989a and 1989b) have shown that when pregnant rats were exposed to 75 parts per million for 7 hours/day from day 7 postpartum to day 21, a significant increase in the density of Purkinje cells was observed in the newborn rats euthanized on day 7 and day 14. This led to an increase in the Purkinje/granule cell ratio of the cerebellum of the developing rat. Furthermore, there were also significant deficits in dendritic arborization and changes in the overall growth process. Selective reductions of cytochrome oxidase (Hayden et al., 1988) and elevations of alkaline phosphatase (Hayden et al., 1987) in similar experiments have also been reported in fetal rat brains. Lastly, in contrast to results obtained from experiments using rats exposed to acute high H_2S concentrations (see below), levels of putative brain amino acid neurotransmitters such as aspartate, GABA, and glutamate were decreased in brains of developing rats when chronic low concentrations of H_2S were applied (Hannah et al., 1988, Hannah et al., 1989c).

F. Studies Investigating the Possible Mechanism(s) of Toxicity Due to Exposure of High Concentrations of H_2S

It has been shown that H_2S is a more potent inhibitor of cellular respiration than hydrogen cyanide, a poison which is believed to have a similar biochemical mechanism of toxicity. Both agents are believed to exert their effects by inhibiting an enzyme, cytochrome oxidase, whose function is crucial for oxidative mitochondrial metabolism to occur (Smith et al., 1977; Nicholls, 1975). The same authors, using particulate preparations of cytochrome oxidase from beef heart, demonstrated that the undissociated form (H_2S) was a more potent inhibitor of the same enzyme than its anionic form (HS^-). In another study, the application of oxygen by inhalation did not significantly prevent sodium sulfide-induced mortality (Smith et al., 1976) yet many victims exhibiting symptoms of systemic intoxication due to H_2S exposure are treated with hyperbaric oxygen (Milby, 1962; Ahlborg, 1951). These findings support the hypothesis that effects seen in acute toxicity from H_2S exposure may be due to inhibition of cytochrome oxidase. The major problem with this hypothesis is that the manifestations, especially those seen in sulfide-induced acute intoxication, occur very rapidly, and therefore, it is unlikely that inhibition of this enzyme would be the underlying cause of these effects seen very shortly after exposure.

In addition, the induction of methemoglobinemia by either sodium nitrate or p-amino propiophenone prior to sulfide application in armadillos, mice, and rabbits provided some protection against the lethal effects of sodium sulfide (Smith and Gosselin, 1964). Smith et al. (1977) also demonstrated that methemoglobin added to a preparation of red blood cells restored the activity of the cytochrome oxidase after it had been inhibited by Na_2S. The utilization of oxidized glutathione, but not reduced glutathione, prior to sodium sulfide administration protected female mice against the lethal effects due to sulfide (Smith and Abbanat, 1966). The protective effects when both oxidized glutathione and methemoglobin were used were additive. Each agent was believed to exert its protective effect through a similar mechanism; they would form a complex with the free sulfide before it would interact with the vital enzymatic processes (Smith and Abbanat, 1966; Beauchamp et al., 1984).

Several enzymes in the CNS have shown to be inhibited by sulfide. These include alkaline phosphatase and adenosine triphosphatase in the cerebellar cortex (Kosmider and Zajusz, 1966; Beauchamp et al., 1984), cerebral cytochrome oxidase (Savolainen et al., 1980), monoamine oxidase (MAO; Warenycia et al., 1989b) and acetylcholinesterase (Maneckjee, 1985). From these findings, it was suggested that the effects of H_2S on the CNS may be due to inhibition of enzymes including those in the respiratory center. It was proposed that the enzyme inhibition was a result of sulfide binding to metal ions required as co-factors (Beauchamp et al., 1984).

Segal (1972) has shown that high concentrations of H_2S inhaled by rats *in vivo* led to blockade of the response in hippocampal granule cells of the dentate gyrus to perforant path stimulation. McLardy (1970) observed sulfide loading in synaptic vesicles containing zinc in the same cells of rats and guinea pigs, however, the enzyme-protein linkage of this zinc was not known. He suggested that the selective blockade of the mossy fiber system in the hippocampus was due to the loading of sulfide in these zinc containing synaptic vesicles.

There is recent evidence to suggest that mechanisms, independent of the poisoning of respiratory enzymes, may be responsible for the effects seen after exposure to high concentrations of sulfide. For example, rapid increases in brain amino acids such as aspartate, glutamate, taurine, GABA, and alanine have been found in the brainstem immediately prior to death induced by intraperitoneal injections of high concentrations of NaHS (Kombian et al., 1988a). In fact, it has been shown that sulfide is selectively accumulated in brainstem (Warenycia et al., 1989a). Also, increases in catecholamines, possibly due to inhibition of monoamine oxidase (MAO), have been observed in the hippocampus, striatum, and brainstem under the same conditions (Warenycia et al., 1889b). Furthermore, the persulfide reagent, dithiothreitol, has been shown to reverse MAO inhibition caused by sulfide (Warenycia et al., 1989c), and to significantly protect whole animals from sulfide-induced lethality (Reiffenstein, 1990). Other studies on bullfrog sympathetic ganglion cells *in vitro* have shown that ligand-gated inhibitory processes (e.g. due to activation of α_2 adrenergic and m_2 muscarinic receptors) or hyperpolarizations produced by exposure to normal potassium concentrations after incubation in low potassium concentrations seem to be potentiated by HS^-; in contrast, excitatory processes (e.g. due to nicotine) are not altered. These studies also suggested an effect of HS^- on the electrogenic Na^+-K^+ exchanger (Kombian et al., 1988). It should be noted that the concentrations of HS^- applied in these experiments far exceeded the LD_{50}. Even very high concentrations of NaHS (e.g. 50 times the LD_{50}) did not alter TTX-sensitive Na^+-channels in mouse neuroblastoma cells (Warenycia et al., 1989d). Application of HS^- (1 to 30 μM) led to complex changes in the firing rate and action potential amplitude recorded extracellularly in the crayfish sensory neuron (Roth et al., 1989), however, intracellular recordings are required to elucidate the mechanism(s) involved.

G. Statement of Purpose

The principle objective of this study was to examine the neurophysiological mechanism(s) by which exposure to high concentrations of H_2S produces neurological toxicity including "knock down" (unconsciousness), and retrograde amnesia upon recovery (Burnett et al., 1977; Report on H_2S toxicity, 1988). Since it is believed that the hippocampus is an important anatomical site for learning and memory (e.g. place memory in rats; O'Keefe and Nadel, 1978) and, because the neurotransmitter identities and pathways of this well understood brain nucleus are well documented, the hippocampus was used as a "model system" to investigate the effects of sulfide on neuronal function. This involved the use of both *in vivo* and *in vitro* electrophysiological recording techniques of CA1 pyramidal cells. The *in* *vitro* slice preparation of hippocampus allows one to overcome many of the problems inherent with the *in vivo* iontophoretic technique coupled with extracellular recording (Hicks, 1984; Dingledine, 1984; Turner and Schwartzkroin, 1984). Furthermore, the hippocampal slice technique gives one greater mechanical stability over *in vivo* recording. This enables one to have better success with intracellular recording, and thus allows for a more detailed analysis of the neuropharmacological investigations involved (Dingledine, 1984). The *in vitro* preparations are also more amenable to rigorous neuropharmacological manipulations. At the same time, the electrophysiological properties of neurons in brain slices (*in vitro*) are similar to those neurons *in vivo*, and the intrinsic and extrinsic synaptic pathways can be preserved intact in slices of hippocampus (Dingledine, 1984). Lastly, but very importantly, the *in vitro* technique allowed for a very careful examination of the mechanism(s) of action of known, toxicologically relevant concentrations of NaHS and to determine whether or not neuronal properties were altered.

CHAPTER II

MATERIALS AND METHODS

I. In Vivo *Experiments*

A. Experimental Animals

Male Sprague Dawley albino rats weighing 240-280 and 75-125 g for *in vivo* and *in vitro* experiments respectively, were obtained from the University of Alberta breeding program (Biological Sciences). Animals were stored in cages with sawdust floors and kept on a 12 hour light-dark cycle at a constant ambient temperature of 24°C. Rat chow ("Wayne rodent blox") and water were accessible to the rats *ad libitum*.

B. Anesthesia

In all experiments, rats were anesthetized by intraperitoneally (i.p.) injected urethane. Usually, the dose applied was 1.25 g/kg, however, in some instances, an increase in the dose to 1.5 g/kg was required to produce a stable lightly anesthetized rat. Urethane is advantageous for these experiments because it has a long duration of action and continuous depth testing and supplementation is not required. Since the animals were incapable of maintaining proper body temperature, a rectal temperature probe, and a heating blanket powered by a 12 V battery connected to a servo control were used to maintain animal body temperature at 37 ± 0.5°C.

C. Surgical Procedures

Blood pressure was monitored in all experiments to ensure that the animal was physiologically stable. PE10 tubing (Clay Adams) was used to cannulate the right femoral artery to record blood pressure. The arterial cannula was connected to a Statham transducer (model P23AA) and heparin in physiological saline (350 units per ml) was used to prevent blood clot formation. Michel wound clips were used to close skin incisions. After placing the animal in a Narashige (SR5) stereotaxic apparatus, a scalpel was used to cut its scalp along the midline and the skin and temporal muscle were moved laterally with a bone raspatory. Some of the scalp muscle was then crushed to help blood clot formation. A hole about 2-3 mm in diameter was drilled in the skull at the proper location so that an electrode could be inserted (see later). After the bone was removed, the dura mater was cut away. The tissue below the hole was kept moist at all times by placing a piece of cotton soaked with physiological saline on it.

D. Stereotaxic Methods

In order to ensure accurate placement of the electrode while the animal was in the stereotaxic apparatus, a reference point was required. This point was calculated by placing a marker at the interaural line and noting the medial/lateral and anterior/posterior coordinates. The following procedure was used to place the animal in the stereotaxic apparatus: earbars were placed in the external auditory meatus and a clamp over the rat's snout was used to hold the incisor bar which was set at 0 mm in height (at the level of the interaural line). The marker was then lowered and if the animal was placed correctly in the apparatus, it would touch the posterior junction of the skull sutures (λ reference point). The marker was then moved to the proper location using λ as the reference point and a cut was made to identify the location where the electrode would descend, and the hole.

CA1 pyramidal neurons of the hippocampus were found 3.8 mm anterior, 1.8-2.2 mm lateral and 2.0-2.6 mm ventral to λ at the surface of the skull.

E. Recording

1. Electrode preparation

The recordings were made from electrodes consisting of five-barreled glass arranged in a cross configuration (thus reducing cross-talk and salt bridge formation). The center recording barrel was composed of thick-walled borosilicate glass (Hillgenberg, O.D. 2 mm, I.D. 1 mm) which contained a glass fiber to facilitate barrel filling by capillary action. The outer drug barrels were comprised of thinner-walled glass (O.D. 1 mm) also containing a glass fiber. The electrodes were designed this way to allow for a center recording barrel of lower resistance and a decrease in the overall capacitance.

The electrodes were constructed in the following manner. A 5-6 cm long recording barrel glass and four drug barrel glasses of various lengths (usually about 0.25-0.5 cm shorter than the recording barrel) were held in a cross configuration by plastic molds that were placed at both ends. Modeling clay was used to hold the outer drug barrels close to the center recording barrel. The pieces were glued at each end with small quantities of quick setting epoxy resin glue. Lastly, a 3-4 mm wide piece of shrink tubing was placed on each end.

While the constructed blank was clamped in a Narishige PE2 electrode puller, the cen[illegible] was heated, twisted a minimum of 360° and allowed to fall 3-5 mm. The heat was then terminated and the magnet and heat adjusted for the pulling of the electrode. The completed electrode was broken back by brushing the tip against a tissue paper until the resistance of the center barrel was 2-10 MΩ. The resistance of the outer barrels was usually 20-80 MΩ. Waterproof color pens were used to label the barrels. After filling the barrels with the appropriate solutions using a 5 cm 32-gauge needle attached to a syringe, silicone grease was applied to the openings to prevent evaporation of the contents and salt bridge formation. The recording barrel contained 2% pontamine sky-blue dye in 0.5 M sodium acetate. In all experiments, 0.15 M NaCl was added to one barrel for current balancing.

2. Equipment

Single unit spikes were recorded extracellularly using a field effect headstage (Dagan) which was connected to the recording barrel via a platinum alloy wire. The signal was carried to an A.C. preamplifier with the gain set at 1000 (Dagan model 2400Z). A 1 μF capacitor was used to couple this output to a 3A9 differential amplifier (Tektronix) of a dual beam cathode ray oscilloscope. The resultant signal was fed into a window discriminator and was then integrated and counted with a rate meter (Ferch electronics). The frequency of the extracellular potential activity was printed with a rectilinear pen system in one channel of a Beckman Type RP dynograph. A loudspeaker which was integrated with the window discriminator was used to monitor extracellular fields so that the oscilloscope would not have to be viewed continuously. A block diagram of the experimental arrangement is shown in Figure 1.

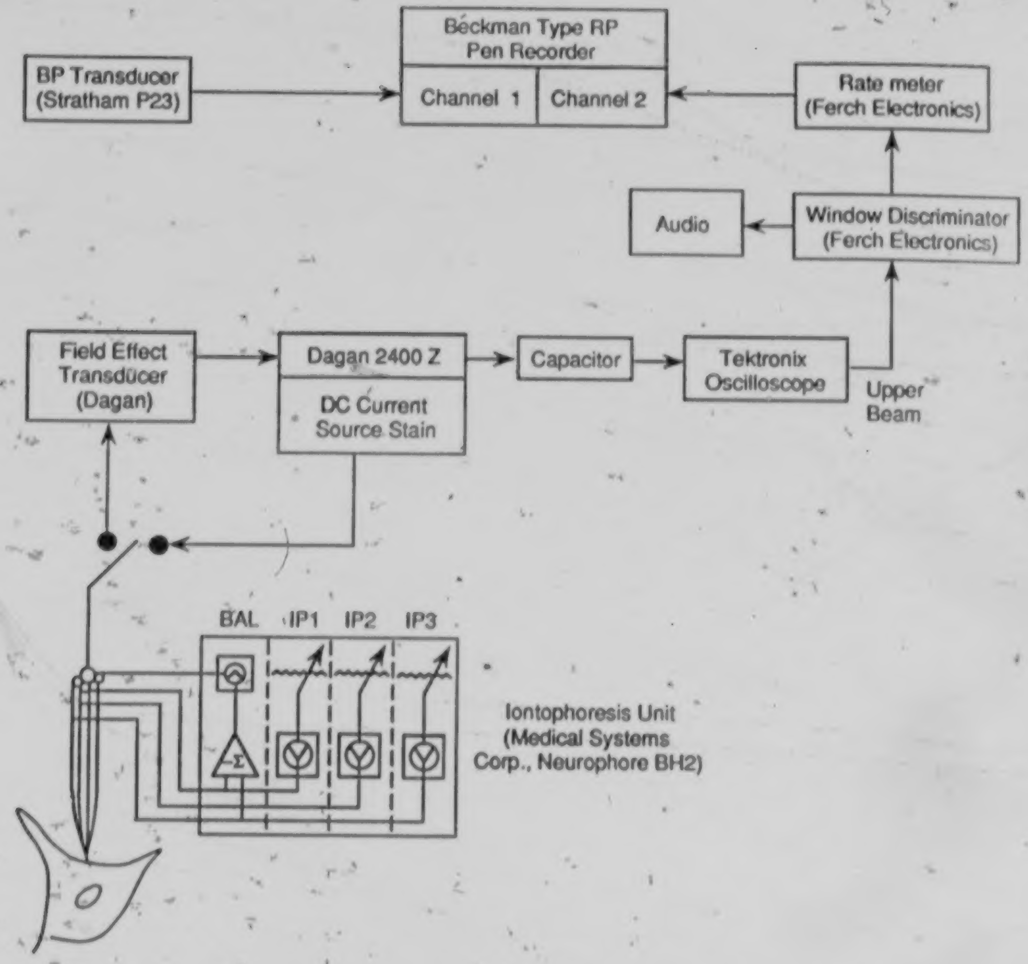

Beckman Type RP
Pen Recorder
Channel 1
Channel 2
BP Transducer
(Stratham P23)
Rate meter
(Ferch Electronics)
Audio
Window Discriminator
(Ferch Electronics)
Field Effect
Transducer
(Dagan)
Dagan 2400 Z
DC Current
Source Stain
Capacitor
Tektronix
Oscilloscope
Upper
Beam
BAL
IP1
IP2
IP3
Iontophoresis Unit
(Medical Systems
Corp., Neurophore BH2)

Fig. 1 Diagram of the experimental arrangement used for extracellular recording and drug iontophoresis *in vivo* (Adapted from Penington, 1986).

F. Physiological Identification of Cells

Single unit spikes were observed on the oscilloscope and discriminated on the basis of spike height. As a result of the application of urethane anesthesia, most cells were either firing at a slow rate or silent. Acetylcholine or glutamate were ejected during experiments using a low current to induce a stable firing rate of 10-20 SPS. This makes it easier to assess inhibitory effects of the agents applied. The CA1 pyramidal cells are easy to locate because they are large, and hence give rise to large field potentials. They also fire with a characteristic bursting pattern; there are several large spikes in each burst and the size of the action potential spike decreases from the first to last spike (Kandel and Spencer, 1961).

G. Drugs and Solutions

All drug solutions were made in double distilled deionized water and adjusted to optimum pH for ionization. Most drug solutions were in the pH 4-8 range, however, to ensure that NaHS was predominantly in the anionic form, a pH of 9 was used. Appropriate controls were performed to ensure that the response obtained was not due to non-specific effects such as high pH (e.g. 0.1M Na_2SO_4, pH 9). Solutions were filtered with 0.22 μm 'GS Millipore' paper to remove small particles. The following drugs and solutions were used:

1. Acetylcholine Cl (Sigma, 0.5 M, pH 5)

2. L-cysteic acid (Sigma, 0.1 M, pH 8.5).

3. Glutamate monosodium salt (MC & B chemicals, 0.1 M, pH 8)

4. Pontamine sky-blue dye (2 % in 0.5 M Na^+ acetate, pH 8)

5. Sodium hydrosulfide ($NaHS{\cdot}xH_2O$, Aldrich Chemical Co., 0.1 M, pH 9)

6. Sodium sulfate (Na_2SO_4, Fisher Scientific Co., 0.1 M, pH 9)

7. Taurine HCl (Sigma, 0.1 M, pH 8.5)

H. Histology

1. Method for recording site marking

The recording electrode was filled with 2% pontamine sky-blue dye in 0.5 M sodium acetate. Since the dye is negatively charged at pH 8, a negative current (10 μA; for 10 minutes) was passed through the recording electrode to eject the dye. This was done at the end of each experiment according to the method used by Hashimoto and Kaneko (1967).

2. Fixation

At the end of the day, the animal was perfused with 10% buffered formaldehyde solution by piercing the left ventricular wall of the rat's heart with a needle. The needle was connected by a rubber tube to a reservoir of formaldehyde and the solution drawn by gravity. After fixation, the brain was removed from the animal and stored in formaldehyde. After some time, the brain was then trimmed, sectioned in a freezing microtome, and the marked site was noted to verify if the recording had occurred in the proper location.

II. In Vitro *Experiments*

A. Preparation and Dissection

Prior to use, all experimental animals were purchased and housed as described above (I-A). Male albino Sprague Dawley rats (75-200 g) were decapitated without prior anesthesia and their brains removed quickly. The brains were rinsed in a beaker containing cold (4°C), oxygenated artificial cerebrospinal fluid (ACSF, pH 7.45), then removed and placed on filter paper resting on covered petri dishes packed with ice. The overlying neocortex was gently parted at the midline, then peeled back and removed to expose the hippocampi. The hippocampi were then separated from the underlying structures and placed on filter papers on the platform of a Stoelting tissue chopper. Slices, 450 μm thick, were cut transversely to the longitudinal axis of the hippocampus. The slices were immediately placed in a beaker containing ACSF which was continuously bubbled with 95% O_2, and 5% CO_2 ("Carbogen"), and were allowed to equilibrate at room temperature until ready to be transferred to the recording chamber.

B. Apparatus

The apparatus used is shown in Plate 1. The entire recording apparatus is mounted on a steel plate placed on tennis balls on the top of an air isolation table

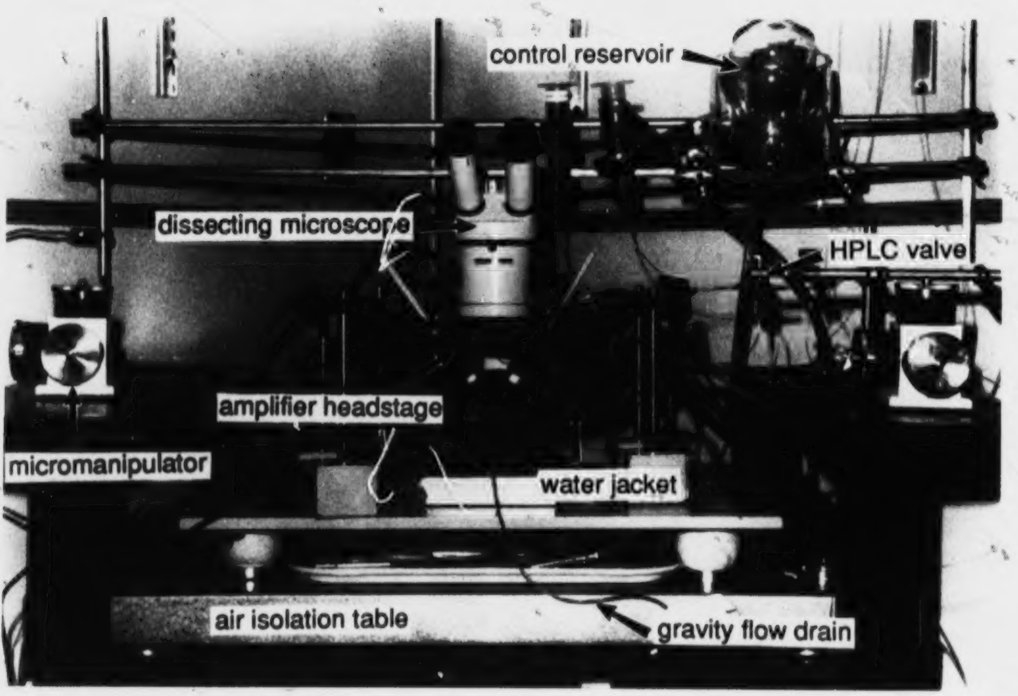

control reservoir
dissecting microscope
HPLC valve
amplifier headstage
micromanipulator
water jacket
air isolation table
gravity flow drain

Plate 1 Experimental apparatus used for electrophysiological recording of area CA1 of rat hippocampal slice.

(Micro-g). The recording chamber itself was mounted on the stage of a Wild M3C dissecting microscope, and illuminated from below, via an adjustable mirror, with a fiber optic light source. The chamber, machined from a single 9 cm by 4 cm piece of 3/16" thick plexiglass, is essentially the same as developed by Henderson and Williams (the "Scottish submersion chamber"; Alger et al., 1984; see Figure 2). The chamber consists of a circular well drilled into the plexiglass within which a nylon mesh is suspended on a 1 mm thick ring cut from the barrel of a 3 ml plastic syringe. The volume of the chamber is about 300 μl. Slices were placed on the nylon mesh, and are held in place with flattened pieces of silver wire.

ACSF was kept prewarmed and bubbled with carbogen in a 1 liter reservoir. A polyethylene (PE-60) tube led from the reservoir to a 4-way HPLC (Hamilton) valve, which permitted rapid, low dead volume switching between source reservoirs. Two other reservoirs (the barrels of 60 ml syringes) were also connected to the switching valve via PE-60 tubing. ACSF drained by gravity via PE-100 tubing through a water jacket, which warmed it to 38°C, and into the chamber. The flow rate was maintained at a constant rate of between 1.5-2.0 ml/min. A change in ACSF at the HPLC valve took about 10 seconds to reach the bath, and the total time required for equilibrium in the empty chamber was about 20-30 seconds. Complete removal of drug from an empty chamber took a total of 45-60 seconds. However, equilibration of drugs into the tissues presumably took longer than this.

ACSF entered the chamber from the rear, below the mesh, and flowed around, beneath and over the slice, which was submerged in ACSF to a depth of

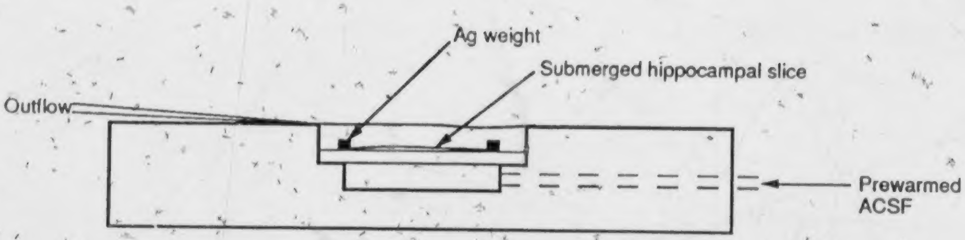

Ag weight
Submerged hippocampal slice
Outflow
Prewarmed
ACSF

Fig. 2 The "Scottish submersion chamber" used for electrophysiological recording of area CA1 of rat hippocampal slice, *in vitro*. (Adapted from Alger et al., 1984.)

about 1 mm. A 3 mm wide, 3 cm long wick of paper (Kimwipe) was placed at the front of the chamber to ensure a constant fluid level; one side of the wick was draped over a Ag-AgCl pellet which served as the chamber ground. A 21 gauge needle, attached to a siphon tube made of Tygon R 3603 tubing, was placed on the wick, centered near the front lip of the chamber. ACSF drained by gravity from the wick to a bucket below by siphon action.

A bipolar stimulating electrode, made from two etched, glass-insulated tungsten rods cemented into the barrel of a Pasteur pipette, was held in a Narashige MM 333 mechanical micromanipulator which permitted selective placement of the electrode in the slice. Amplifier headstages were mounted on the headstages of Narashige MO 103 hydraulic micromanipulators which were in turn firmly mounted on magnetic bases to the steel plate. The microelectrode holders were plugged directly into the headstages.

C. Electrophysiological Recording

The experimental arrangement of the electrodes used for electrophysiological recording *in vitro* is shown in Plate 2. The arrangement of the electrodes in the slice is shown in Figure 3. The stimulating electrode was placed in *stratum radiatum* near the CA2-CA3 boundary to elicit orthodromic activation of the Schaffer collateral projection to CA1 pyramidal cells. Stimuli were supplied by a stimulus isolating unit (A.M.P.I.).

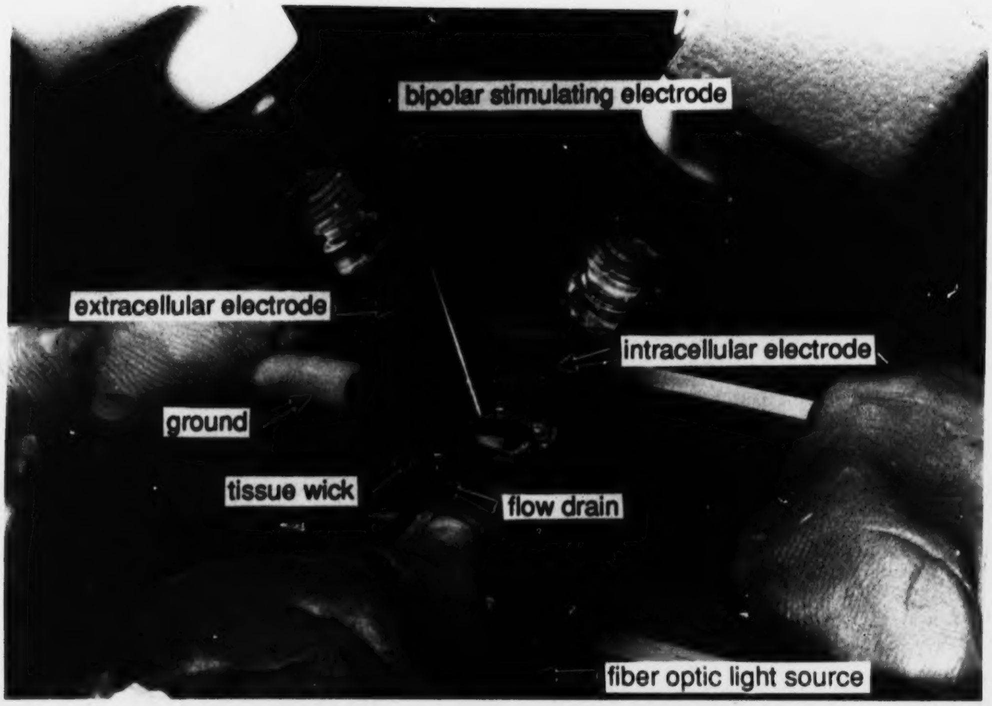

bipolar stimulating electrode
extracellular electrode
intracellular electrode
ground
tissue wick
flow drain
fiber optic light source

Plate 2 Experimental arrangement of electrodes used for electrophysiological recording of area CA1 *in vitro*.

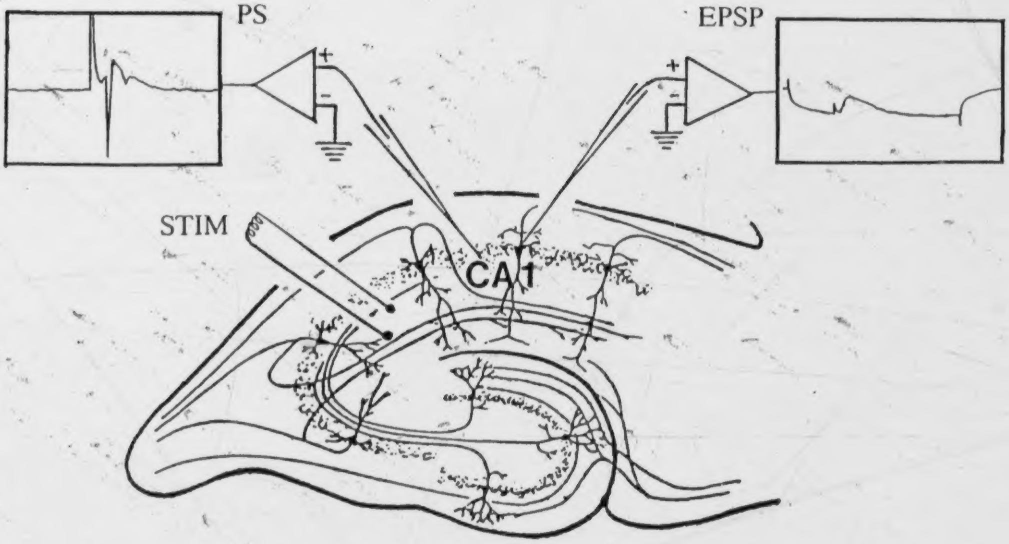


Fig. 3 A schematic diagram of a hippocampal slice showing a bipolar stimulating electrode placed in *stratum radiatum* to activate CA1 pyramidal cells orthodromically. An extracellular recording electrode is used to record (upper left) the compound field potential and population spike (PS) arising from the stimulation. On the right is shown the simultaneous intracellular recording from an impaled pyramidal cell in which the excitatory postsynaptic potential (EPSP) can be seen following *stratum radiatum* stimulation. The EPSP is augmented in this case by superimposing a hyperpolarizing pulse via a bridge circuit. Figure adapted from Pittman et al. (1987).

1. Extracellular recording

Extracellular electrodes were pulled by a Flaming/Brown Micropipette Puller (Model P-87) from thin-walled glass (1.0 mm O.D.). Filled with NaCl (2 M), they had d.c. resistances of 2-6 MΩ. The electrodes were placed in the *stratum pyramidale* of area CA1 and advanced until a maximum field response to a given electrical stimulus to *stratum radiatum* was elicited. The field potential was composed of 2 wave components. The first was a slow, positive-going wave, which reflects the time course of the dendritic depolarization of the CA1 cells (Andersen et al., 1971a). The second component, a sharp negative wave, was superimposed on the positive wave, and represents the synchronous action potential discharge of many CA1 pyramidal cells (Andersen et al., 1971 a, b). This component is called the population spike (PS) and has the same time course as the intracellularly recorded action potential (Andersen et al., 1971a). The stimulus voltage was varied systematically from near threshold for the PS to the maximum response amplitude. Most stimuli were presented at a voltage chosen to elicit a response of between 50 and 75% of maximum (i.e. on the linear portion of the stimulus intensity-curve; Andersen et al., 1971b; Lømo, 1971) so that any changes (e.g. increases or decreases) in the responses due to application of the various agents including H_2S would be clearly evident. The electrode was connected via the headstage to channel 2 of the amplifier (Axoclamp 2A). This unity gain signal output was connected to a Grass P15 amplifier with the gain set at 10.

In some experiments, a second extracellular electrode was inserted into the apical dendritic region of area CA1 to record simultaneously the field excitatory postsynaptic potential (EPSP) evoked by *stratum radiatum* stimulation (Andersen et al., 1980; Colmers et al., 1987). The field EPSP reflected the EPSP's generated in the apical dendrites of a population of CA1 neurons (Andersen et al., 1978). The electrode was connected via the headstage to channel 1 of the Axoclamp.

2. *Intracellular recording*

Intracellular electrodes were pulled on the P-87 from 1 mm diameter thick-walled glass, filled with potassium acetate (KAc; 2 M), KCl (2 M), or CsCl:CsAc (4:1; 2 M) and placed in the cell body layer of area CA1. The electrodes had d.c. resistances of 80-160 MΩ. Cells impaled were identified as pyramidal neurons using criteria including action potential amplitude and duration, and the response to *stratum radiatum* stimulation (Andersen et al., 1980, Colmers et al., 1987). Cells whose resting membrane potential was more negative than -55 mV, stable for at least 20 minutes, and whose action potential amplitudes were greater than 85 mV, were considered acceptable. The electrodes were connected, via the headstage, to channel 1 of the Axoclamp, used in the bridge current clamp mode. Bridge balance was closely monitored throughout the experiments. To avoid generation of action potentials, excitatory postsynaptic (EPSP) responses from single neurons were elicited during a membrane hyperpolarization (125 ms duration; Colmers et al.,

1987) induced by the injection of a negative current pulse of constant amplitude through the electrode via the bridge current clamp circuit of the amplifier. The stimulating voltages applied to *stratum radiatum* ranged from near threshold for the EPSP to the maximum amplitude possible without the neuron reaching action potential threshold. Most stimuli were presented at voltages chosen to elicit responses between 50 and 75 % of maximum.

The timing of all experiments was controlled by a Master-8 (A.M.P.I.), an 8-channel programmable pulse generator. For example, once the cell was acquired, the Master-8 was programmed to trigger the oscilloscopes, generate the hyperpolarizing (or negative) current pulses (125 ms) via the Axoclamp and 40 ms after the initiation of each of these pulses, a square wave, monophasic pulse stimulus to the bipolar electrode triggered via the stimulus isolating unit. This cycle of hyperpolarizing and stimulating pulses occurred once every 10 seconds (0.1 Hz). For assessment of neuronal input resistance, 125 ms square wave, hyper- and depolarizing current pulses were generated by the Master-8 at 1 Hz. The amplitudes of the current pulses applied for estimation of input resistance were manually controlled with an attenuator which could vary current command amplitude in steps of 0.1 nA.

In some experiments, CA1 pyramidal neurons were impaled with electrodes filled with KAc (2 M) having resistances relatively lower (e.g. 60-80 MΩ) than those described above. After stable recordings were obtained, the Axoclamp was set to the discontinuous single-electrode voltage clamp (SEVC) mode. Switching frequencies were set between 3.2 and 4.0 kHz. At the beginning of each experiment, the amount of capacitance compensation was set, and the headstage output was monitored closely on a separate oscilloscope to ensure clamp stability and to prevent clamping artifacts due to incorrect adjustments (i.e. clamping of the electrode). The clamp gain was gradually increased to a point, consistent with stability. Neurons were clamped at their resting potential. Slow voltage command ramps (1 mV/sec) were applied and the cells were slowly depolarized from -120 mV potential to -40 mV; simultaneously, current-voltage (I-V) plots were constructed directly on an *x-y* plotter (Hewlett Packard Model 7035B).

All current and voltage signals from intracellular recordings and voltage signals from extracellular recordings were delivered to a patch panel from which they were connected to the analytical instrumentation. Data were collected and stored for on- and off-line analyses. A Nicolet 4094 digital oscilloscope was used to analyze and record on floppy diskettes averaged PSs and EPSPs, action potentials, and the individual current pulses used to either depolarize or hyperpolarize the cell. A DC-coupled pen recorder (Gould 3200S) displayed a continuous record of membrane potential and injected currents. All signals were digitized (Neurocorder, model DR 886; input gain ± 2V full scale) and stored on videotape (Sharp XA-300) for offline analysis with the Nicolet.

3. Iontophoresis experiments

In some experiments, glutamate was applied iontophoretically to the dendrites of an impaled neuron in slices in which a stimulating electrode was placed in *stratum radiatum*. A thin-walled glass microelectrode, filled with 0.25 M-sodium glutamate at pH 8.0., resistance 4-10 MΩ, was lowered into the apical dendritic area of CA1 pyramidal neurons. The glutamate electrode was connected via a headstage (Axon Instruments HS2 - 1.0L, which permitted passage of up to ± 200 nA) to channel 2 of the Axoclamp. The Master-8 was programmed to generate negative current pulses (10-15 ms, 0.2 Hz) via the Axoclamp, and the amplitudes of the current pulses (80-120 nA) applied for glutamate ejections were manually controlled with the step command thumb-wheel of the Axoclamp. During interpulse intervals, retaining currents of +10 nA were used to prevent diffusion of glutamate from the electrode. After impaling a pyramidal neuron, the glutamate electrode was advanced through the dendritic layer of area CA1 until a maximum response (e.g. depolarization) to the iontophoretic pulses was observed in the neuron. Once stable and reproducible responses to the glutamate applications were observed, experiments commenced. A block diagram of the experimental arrangement is shown in Figure 4.

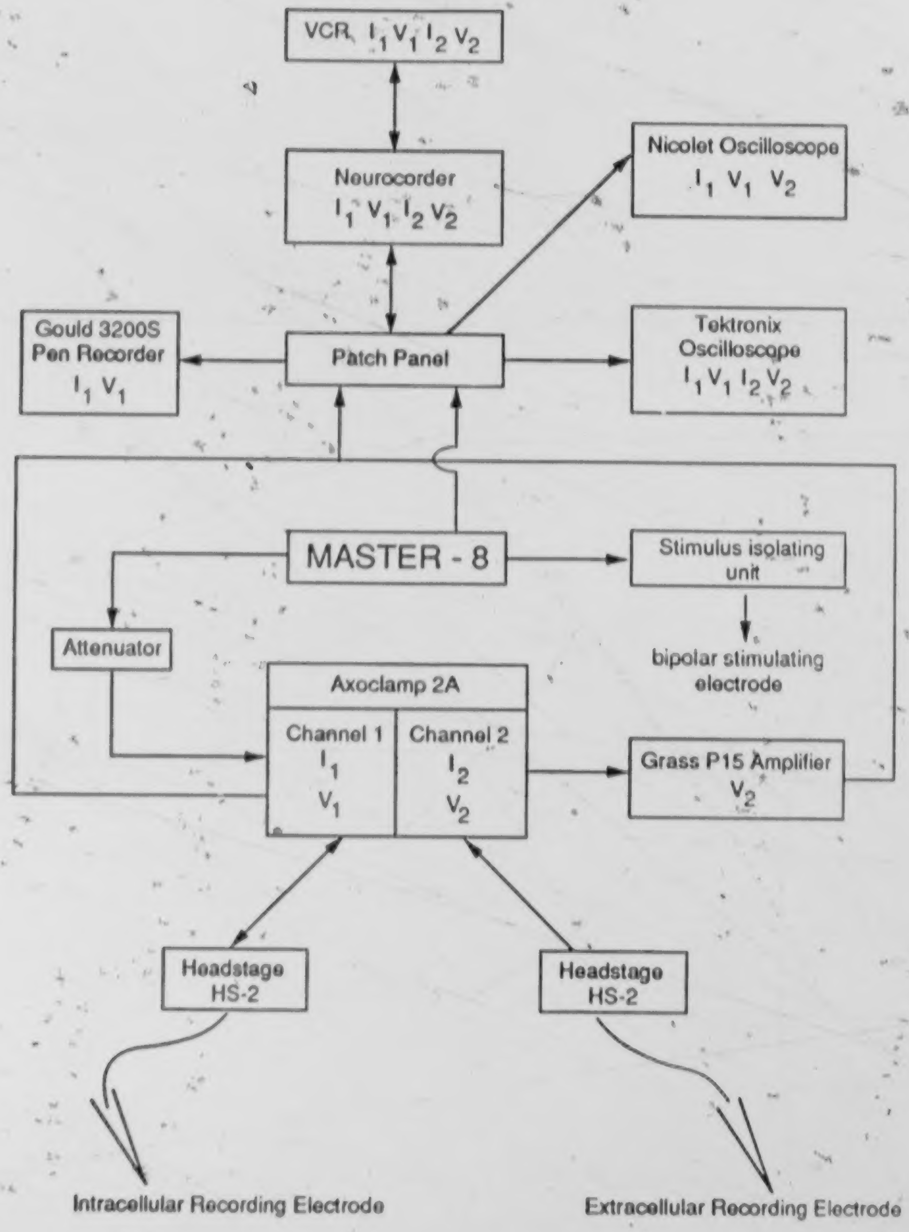

VCR I_1 V_1 I_2 V_2
Neurocorder I_1 V_1 I_2 V_2
Nicolet Oscilloscope I_1 V_1 V_2
Gould 3200S Pen Recorder I_1 V_1
Patch Panel
Tektronix Oscilloscope I_1 V_1 I_2 V_2
MASTER - 8
Stimulus isolating unit
bipolar stimulating electrode
Attenuator
Axoclamp 2A
Channel 1 I_1 V_1
Channel 2 I_2 V_2
Grass P15 Amplifier V_2
Headstage HS-2
Headstage HS-2
Intracellular Recording Electrode
Extracellular Recording Electrode

Fig. 4 Diagram of the experimental arrangement used for extracellular and intracellular recording and glutamate iontophoresis in vitro. ("VCR" = video cassette recorder (Sharp XA-300); "Master-8" = pulse generator; Axoclamp 2A = amplifier.)

D. Preparation of Artificial Cerebrospinal Fluid

ACSF solutions were made by dilution of a 10 times stock solution of most salts; $NaHCO_3$ and glucose were added to the dilute solution. The final composition of the ACSF contained: NaCl, 124 mM; $NaHCO_3$, 26 mM; glucose, 10 mM; $MgSO_4$, 2 mM; KCl, 1.8 mM; $CaCl_2$, 1.5 mM; and KH_2PO_4, 1.25 mM. All drugs were added hyperosmotically to the ACSF.

E. Preparation of NaHS and Other Reagents

Drugs were prepared as concentrated stock solutions in Milli "Q" purified water (except strophanthidin which was prepared in absolute ethanol) and dissolved to final concentrations in carbogenated ACSF immediately prior to use. The following drugs were used:

1. Sodium hydrosulfide, $NaHS\text{-}xH_2O$, Aldrich Chemical Co.
2. Tetraethylammonium chloride (TEA)
3. 4-aminopyridine (4-AP)
4. Cesium chloride (CsCl)
5. Tetrodotoxin (TTX)
6. Strophanthidin

Furthermore, the reagents which were applied intracellularly via the electrode, were prepared as concentrated stock solutions in purified water and dissolved to final concentrations in potassium acetate (2 M) before filling the electrodes. The one exception was in experiments involving electrodes filled with cesium chloride: cesium acetate (4:1; 2 M). These reagents included:

1. 1-,2 bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA; tetra-potassium salt)
2. Adenosine 5'-triphosphate (ATP; disodium salt)
3. Cesium chloride:cesium acetate (4:1; 2 M)

All drug concentrations are expressed in terms of their salts and were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise noted. Stock solutions of TEA, 4-AP and TTX were stored at 0-4°C prior to use, whereas concentrated solutions of strophanthidin, ATP and BAPTA were kept below 0°C before use. All NaHS solutions were made fresh daily.

F. Construction of Concentration-Response Curves for NaHS

At pH 7.45, whether gaseous H_2S or NaHS was originally the source, approximately one-third exists as H_2S and two-thirds exists as HS^- (Beauchamp et al., 1984). Concentrations of NaHS applied to the slice were chosen based on those found in brains of rats acutely exposed to H_2S gas or given intraperitoneal injections of NaHS. At the LD_{50}, brain acid-labile S^{2-} was found to be 75 μM

(Warenycia et al., 1989a; Goodwin et al., 1989). Therefore, in these experiments concentrations of NaHS of between 27 and 200 μM were applied to the preparations for durations of between 3.5 and 6 minutes to ensure an equilibrium response. Sulfide solutions were washed out with control ACSF for at least 20 minutes to ensure substantial recovery (e.g. at least 95 % recovery in EPSP amplitude) before application of the next concentration of NaHS. During experiments involving extracellular recording, increasing concentrations of NaHS were applied successively with intervening and adequate recovery periods until the desired concentration range of NaHS was completed. A similar protocol for experiments involving intracellular recording was followed except that only portions of this concentration range of NaHS could usually be applied to the same cell because successful intracellular recordings of these cells were rarely long enough for application of a complete concentration range.

G. Data Analysis

1. Measurement of changes in membrane potential

For HS^- applications, membrane potential changes (e.g. hyperpolarizations) were measured from the resting membrane potential observed preceding the application, to the maximum change in membrane potential seen during the application. The "washout hyperpolarization" was calculated as the maximum difference in membrane potential observed during the wash from the resting membrane potential preceding the NaHS application.

2. Measurement of neuronal input resistance

As mentioned above (section C,2), families of hyper- and depolarizing 125 ms current pulses were applied at 1 Hz to the neuron before, during, and after drug applications and used to estimate neuronal input resistance. Voltage responses were taken as the maximum voltage deflection elicited by the constant current pulse. These values were plotted against current pulse amplitudes and the neuronal input resistance estimated from the slope of a least-squares regression line fitted to the data (Colmers et al., 1987; 1988).

3. Measurement of PS and EPSP amplitudes

PS amplitudes were measured from the peak of the first negative wave to the peak of the succeeding positive wave (Colmers et al., 1985; 1987). The amplitudes of the EPSP's were measured from the membrane potential just after the stimulus artifact to the peak amplitude of the EPSP (Colmers et al., 1987).

4. Statistics

All data were obtained from neurons which showed substantial recovery (e.g. at least 95 % recovery in EPSP amplitude) from the drug effects. All data are presented as means ± standard errors of the mean (SEM). Neurons were used as their own controls for most statistical analyses. Comparisons were made against the immediately preceding sulfide-free responses using Student's paired-t test. Because no such controls were possible with neurons impaled with electrodes filled with other than the standard electrolyte solution, the comparisons were made with responses observed at identical HS^- concentrations in neurons impaled with electrodes containing only 2 M KAc using a t-test for 2 means (Dowdy and Wearden, 1983). Statistics tables by Fisher and Yates (1957) were used to determine values of probabilities. Probabilities below 0.05 were considered statistically significant.

CHAPTER III

RESULTS

I. In Vivo *Experiments*

The objective of this part of the study was to examine the effects of HS^- on the firing rate of hippocampal CA1 pyramidal cells of the intact rat (*in vivo*). This model allowed for the investigation of the effects of HS^- under ideal conditions (except for anesthesia) in which all synaptic pathways connecting the hippocampus with other areas of the cortex were both physically and physiologically intact as opposed to the *in vitro* hippocampal slice where this was not the case. Furthermore, it has been demonstrated that sulfur-containing amino acids such as taurine or cysteic acid, or HS^- alone, do not alter the excitability of mouse neuroblastoma cells, while either amino acid, together with HS^-, completely block tetrodotoxin-sensitive Na^+ channels (i.e. action potentials; Warenycia et al., 1989d). In light of this, some experiments were undertaken to assess the effects of HS^- in combination with taurine or cysteic acid.

A. Effects of Acetylcholine and Glutamate

Stable recordings were obtained from 33 hippocampal pyramidal cells. The recording sites were verified to be in area CA1 via histological analysis. Since the CA1 cells were silent in the anesthetized animals, continuous ejection of small amounts of either ACh or GLU (1-15 nA) was usually applied to obtain a relatively high (e.g. 3-5 SPS) and stable cell firing rate; in some experiments, it was not necessary to apply retaining current in the ACh-containing barrel to allow for slow diffusion of ACh from the electrode tip. In several experiments in which the cell firing rate due to ACh leakage or ejection was still low (e.g. less than 3 SPS), and therefore it was difficult to show if any inhibition was occurring, large pulses of glutamate (e.g. 20-40 nA) were applied for 30 second time intervals with intervening periods between glutamate pulses lasting for 2-3 minutes or until recovery occurred. Application of the glutamate pulses elicited pronounced increases in cell firing rates which often reached its maximal rate slightly before the end of the 30 second pulse interval.

B. Effects of Iontophoretically Applied NaHS

In 25 cells in which a stable firing rate was maintained with a constant ejection of small amounts of ACh or GLU, HS^- application suppressed the firing rate of 15 of these cells. Of the other 10 cells, HS^- caused an increase in firing rate in 5, while 2 were unaffected; in the 3 remaining cells, HS^- application led to an increase in cell firing initially, but a decrease (below baseline firing rate) was observed afterwards during continuous application of HS^-. Application of ejection currents of Na_2SO_4 (controls) similar to those of HS^- in 7 of these cells did not alter cell firing rate (see Figure 5). One important observation is that most cells (e.g. 22 of the 25 cells) may be treated with HS^- many times, to the point of total inhibition of firing, with complete reversibility of activity occurring within 3-5 minutes.

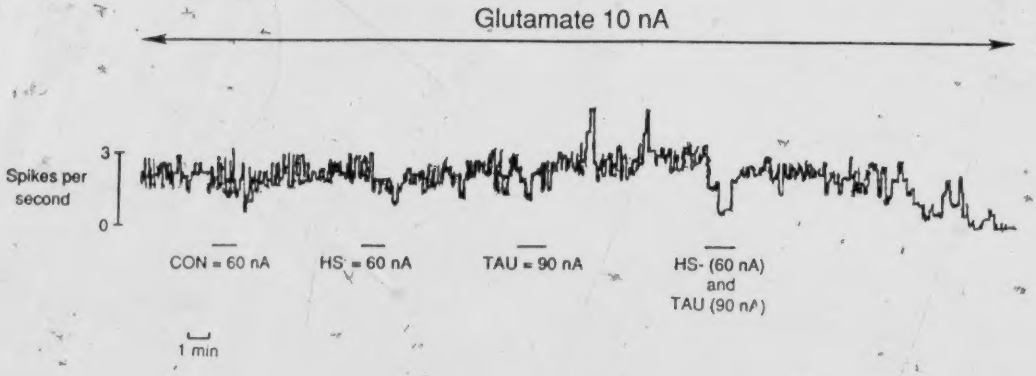


Fig. 5 Effect of HS$^-$, taurine (TAU), and both together on firing rate of hippocampal CA1 neuron *in vivo*. Firing of a pyramidal cell is inhibited reversibly by iontophoretic application of HS$^-$ (while no change in cell firing rate occurred in response to application of Na_2SO_4, control (CON) and TAU alone. However, the combined application of both TAU and HS$^-$ led to a much larger, irreversible inhibition and apparent death of the cell.

In 13 cells in which cell firing was increased by application of glutamate pulses, the firing rates of all of these cells were suppressed by ejection of HS$^-$ and glutamate simultaneously as compared to the glutamate controls (see Figure 6B).

C. Effects of Sulfur Containing Amino Acids

As mentioned above, results from experiments in neuroblastoma cells *in vitro* suggest that a synergistic action of HS$^-$ with the sulfonated amino acids, taurine or cysteic acid, may occur (Warenycia et al., 1989d). Taurine is a neuroinhibitory amino acid which is found endogenously, and it has been suggested that taurine opens Cl$^-$ channels (Champagnat et al., 1982; Curtis and Watkins, 1960; 1965). Cysteic acid, on the other hand, is an excitatory precursor of taurine which is thought to exert its effects through a stimulation of cAMP formation (Baba et al., 1982; Curtis and Watkins, 1960; 1965). In order to test the hypothesis that the combination of HS$^-$ with taurine or cysteic acid leads to a greater effect than would be observed if each was applied alone, HS$^-$ was applied in the presence of taurine or cysteic acid in some experiments, and the effects on cell firing rate were observed.

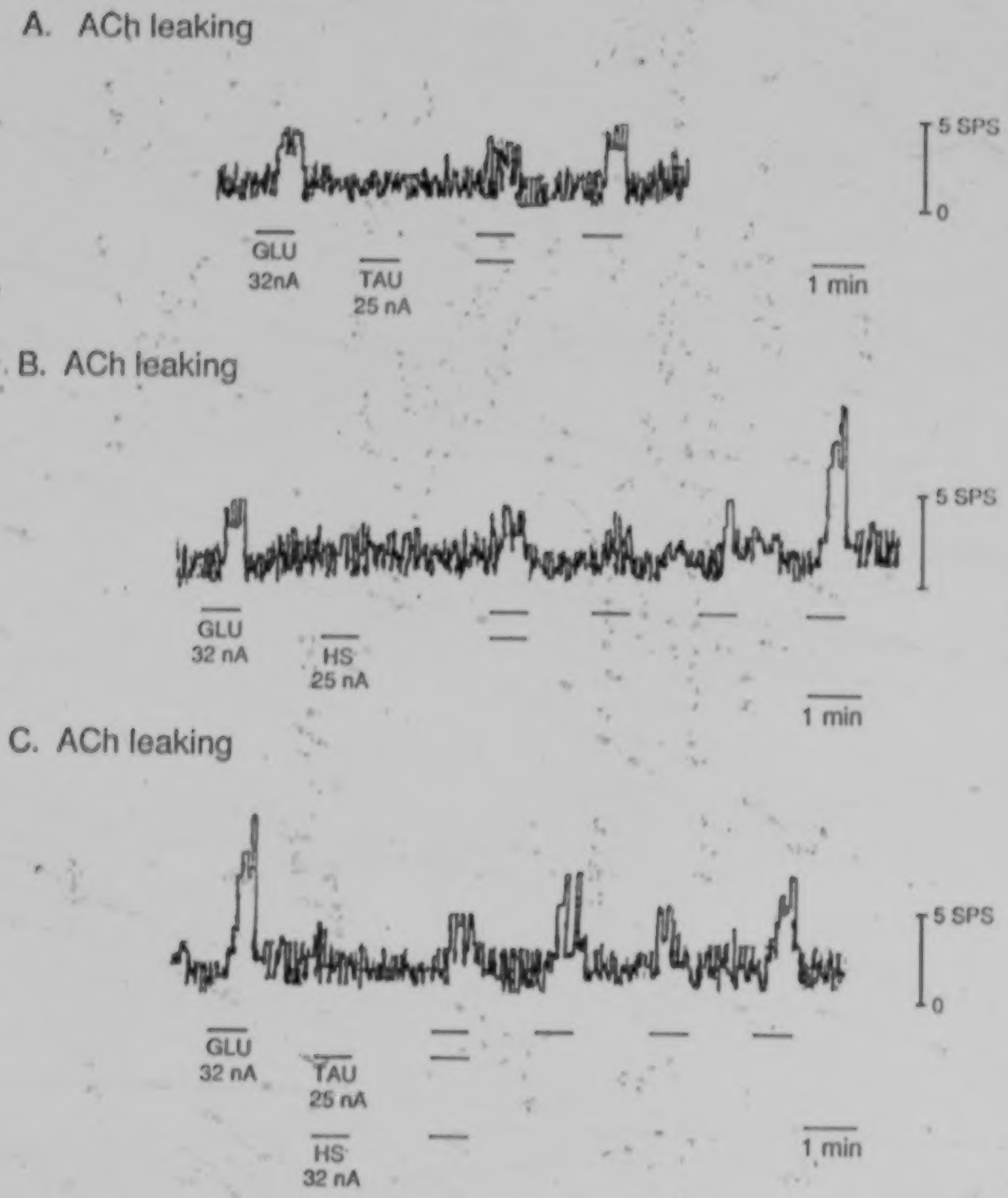
Although application of HS$^-$ and taurine alone inhibit the firing rate of spontaneously active or glutamate-driven CA1 cells reversibly (see Figure 5 and 6 A and 6B), the ejection of both these agents simultaneously leads to a larger, often irreversible, inhibition or apparent death of the cell; for example, these combined applications led to irreversible damage or apparent death in 8 of 25 cells examined (e.g. see Figure 5 and 6C).

Fig. 6 Hippocampal CA1 neuron firing and the effect of HS^-, taurine (TAU), and both together on responses to glutamate (GLU) pulses. Acetylcholine (ACh) was freely diffusing from the pipette throughout.

A. TAU reversibly inhibited the increase in firing rate due to application of glutamate. TAU (alone) had no effect.

B. HS^- reduced the glutamate-induced increase in firing rate while application of HS^- alone had little effect. The response due to application of glutamate alone did recover completely 6-7 minutes after application of HS^- and glutamate.

C. The application of both, TAU and HS^-, in the absence of glutamate, had little effect. However, TAU and HS^- applied together caused a larger decrease in the glutamate-induced increase in firing rate than caused by TAU (A) or HS^- (B) separately. Furthermore, the response to glutamate ejection, after application of TAU and HS^-, never recovers completely to that of controls. (SPS = spikes per second).



A. ACh leaking

B. ACh leaking

C. ACh leaking

The ejection of cysteic acid led to an increase in the firing rate of all 12 cells so studied. HS^- application, alone, led to a decrease in the baseline firing of only 4 of these cells, while in the remaining 8 cells, the firing rate of 2 cells were actually increased, and the rest were relatively unchanged. However, it should be mentioned that the baseline firing rate of these cells was often very low (e.g. about 1 SPS, in the absence of cysteic acid). Of the 10 cells in which the application of HS^- alone did not lead to an increase in the cell firing rate, application of HS^- in the presence cysteic acid led to larger decreases in cell firing rate compared to HS^- alone in 7 of these cells. For the other 3 cells, the degree of inhibition caused by HS^- was the same in the presence or absence of cysteic acid. For the 2 cells in which both HS^- and cysteic acid led to increases in cell firing rates, the combined application of both agents led to an increase somewhere between the responses obtained for the application of each agent alone.

D. Discussion

From these results, it was difficult to conclude precisely the mechanism(s) by which HS^- may have been exerting its effects. It seemed that HS^- inhibited both the spontaneous and glutamate-driven activity of the CA1 pyramidal cells reversibly; however, in some cells, ejection of HS^- actually led to an increase in the firing rate. There are some plausible explanations for these discrepancies. For example, since a finite volume of tissue was treated during each ejection of HS^-, this may have caused a stronger inhibition of a nearby inhibitory interneuron than the CA1 neuron that was being recorded, and thus, may have led to an increase in the firing rate of the recorded pyramidal neuron. Other possible explanations for these inconsistent observations may have included: (1) since the concentrations of HS$^-$ applied were not known, each cell was probably exposed to different concentrations of HS$^-$, hence, HS$^-$ could have exerted different effects since the various doses would have represented different levels at the dose-response curve; (2) the firing rate of some of the recorded neurons could have been affected by the high pH of the HS$^-$ solution applied, although this was unlikely because applications of controls (Na_2SO_4, same pH) to some of the neurons did not alter firing rates; and (3) in some cells, when ejection of HS$^-$ resulted in initial excitation which was followed by inhibition, this may be attributed to barrel warm-up, that is, the amount of HS$^-$ ejected increased over time when the iontophoresis began (Hicks, 1984). However, interestingly, many of the cells could be treated with HS$^-$ several times, which often led to substantial changes in the activity of these cells, yet the cells often recovered completely and rapidly.

When HS$^-$ was ejected in combination with taurine, a larger, often irreversible, inhibition was observed. However, based on these results, we do not know if this inhibition was an additive effect due to the activation of identical inhibitory processes or due to a further mechanism independent of the putative neurotransmitter role (excitatory or inhibitory) of taurine. For example, application of HS$^-$ and taurine together (but not alone) blocked TTX-sensitive Na^+ channels in mouse neuroblastoma cells *in vitro*, and thus would have a profound effect on on AP generation (Warenycia et al., 1989d). Since it has been reported that taurine binds to a receptor and activates a Cl^- conductance in the cerebellum (Sakai et al., 1985), they suggested that taurine was increasing the permeability of the neurons to anions such as HS^-, and that this interaction may be involved in H_2S toxicity.

In the experiments in which the effects of HS^- and cysteic acid applied in combination were examined, it is difficult to conclude whether or not the application of HS^- and cysteic acid led to an enhancement of the sulfide-mediated effects. This is so especially since in the absence of cysteic acid, the baseline firing rates of most cells were low and therefore, it was difficult to demonstrate if HS^- (alone) had any inhibitory effects on neuronal activity, and furthermore, ejection of HS^- to some cells actually resulted in excitation of these neurons.

The most important finding was that individual neurons were not irreversibly inhibited by sulfide, but were able to completely recover after many, prolonged applications of sulfide. This suggests the possibility that in whole animals, apparent anoxic effect of H_2S poisoning and subsequent brain death result from the impairment of breathing, rather than impairment of oxygen utilization by brain cells. It also suggests that *immediate* mechanically assisted respiration may reverse poisoning in a substantial number of victims.

Because of the limitations associated with the iontophoresis and extracellular recording techniques, we decided to examine the effects of HS^- on CA1 neurons *in vitro*. By using the intracellular recording technique *in vitro*, this would enable us to eliminate many of the ambiguities seen during extracellular recordings. Moreover, this would allow us to investigate the effects of HS on active membrane properties (e.g. action potential amplitude and duration) and passive membrane properties (e.g. resting membrane potential and resting input resistance) and thus to determine more precisely the molecular mechanism(s) that may be involved in H_2S toxicity.

II. In Vitro *Experiments*

In order to examine the mechanism of action of H_2S in greater detail, we studied the effects of toxicologically relevant concentrations of HS^- on CA1 pyramidal cells in the *in vitro* slice preparation of rat hippocampus.

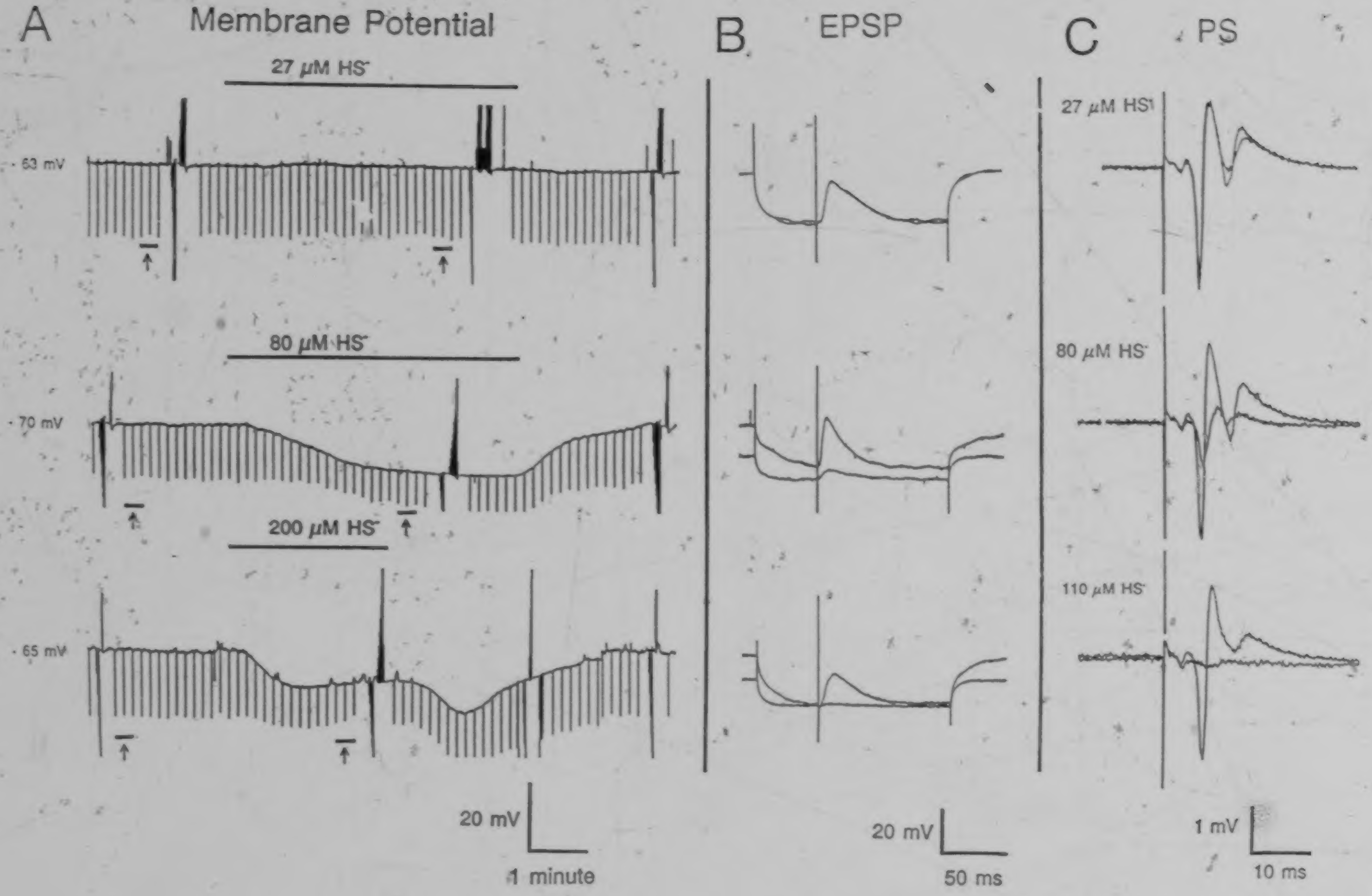
Results were obtained from applications of NaHS to 81 CA1 pyramidal cells under different experimental circumstances. Neurons used in this study satisfied the following minimum criteria: resting membrane potential more negative than -55 mV, slope input resistance greater than 35 MΩ, action potential amplitudes greater than 85 mV, and action potential overshoot greater than 25 mV. The properties of the impaled CA1 pyramidal neurons in these experiments were similar to those reported by others in CA1 cells *in vitro* (Turner and Schwartzkroin, 1984; Colmers et al., 1987).

A. Effects of NaHS on Membrane Properties and Responses

1. Initial membrane hyperpolarization and changes in input resistance

The application of NaHS (≥ 80 μM) caused a rapid (0.5-1 minute onset) membrane potential hyperpolarization that was associated with a decrease in neuronal input resistance (Figure 7A). These effects were dependent upon the

Fig. 7 Effect of different concentrations of HS^- on membrane potential, excitatory postsynaptic potential (EPSP) and orthodromically-evoked extracellular population spike (PS) amplitudes in CA1 pyramidal neurons. A. Chart records of membrane potential, and B. digital oscilloscope traces at an expanded time scale of EPSP's evoked during brief (125 ms), constant current hyperpolarizing pulses. Concentrations of HS^- greater than 60 μM caused membrane hyperpolarizations, which reversed rapidly upon washout. Concentrations of $HS^- \geq 110$ μM led to a brief "washout" hyperpolarization prior to complete recovery (Lower trace A). B. Digital averages of 3 successive stimuli (arrows in A). HS^- (≥ 60 μM) reduced EPSP amplitudes, by a maximum of 81% (HS^- concentration = 200 μM; lower trace, B). C. Digital averages of 5 successive PS's, evoked orthodromically in CA1 by *stratum radiatum* stimuli. Slice preparations were different than in A and B. Control and sulfide traces are superimposed. Concentrations of $HS^- \geq 27$ μM reduced PS amplitudes; $[HS^-] \geq 110$ μM reversibly abolished PS altogether.


A
Membrane Potential
27 μM HS⁻
80 μM HS⁻
200 μM HS⁻
- 63 mV
- 70 mV
- 65 mV
20 mV
1 minute
B
EPSP
20 mV
50 ms
C
PS
27 μM HS⁻
80 μM HS⁻
110 μM HS⁻
1 mV
10 ms

NaHS concentrations used; for example, NaHS ($\geq$ 40 μM) caused a significant membrane potential hyperpolarization ($p < 0.02$, n=11; $EC_{50} \simeq 90$ μM, see Figure 8A); with concentrations of NaHS $\geq$ 80 μM, a significant decrease in membrane resistance was observed ($p < 0.05$, n=13) up to a maximum of 35% at 160 μM ($p < 0.01$, n=7; $EC_{50} \simeq 115$ μM, Figure 8B). The voltage-current (V-I) relationship, determined in current clamp, of the CA1 pyramidal cell of Figure 7 is shown in Figure 9A. Application of 80 μM of NaHS (the brain sulfide concentration found in rats exposed to the LD_{50} (Goodwin et al., 1989; Warenycia et al., 1989a), caused a downward shift, reflecting the hyperpolarization, and a decrease in the slope of this line, indicating a decrease in membrane resistance. All of these effects were rapidly reversible, usually requiring 2-10 minutes wash for complete recovery.

The reversal potential for the HS^--induced conductance change was estimated from the intersection of V-I lines obtained with brief current pulses during current clamp (Kuffler et al., 1984; Leblond and Krnjević, 1989). In some cases, the point of intersection had to be determined via extrapolation of the V-I lines since this point was outside the range of the plotted values. The reversal potential for the conductance change mediated by application of 200 μM HS^- in 18 cells was about 99.8 ± 5.0 mV (see Figure 9B).

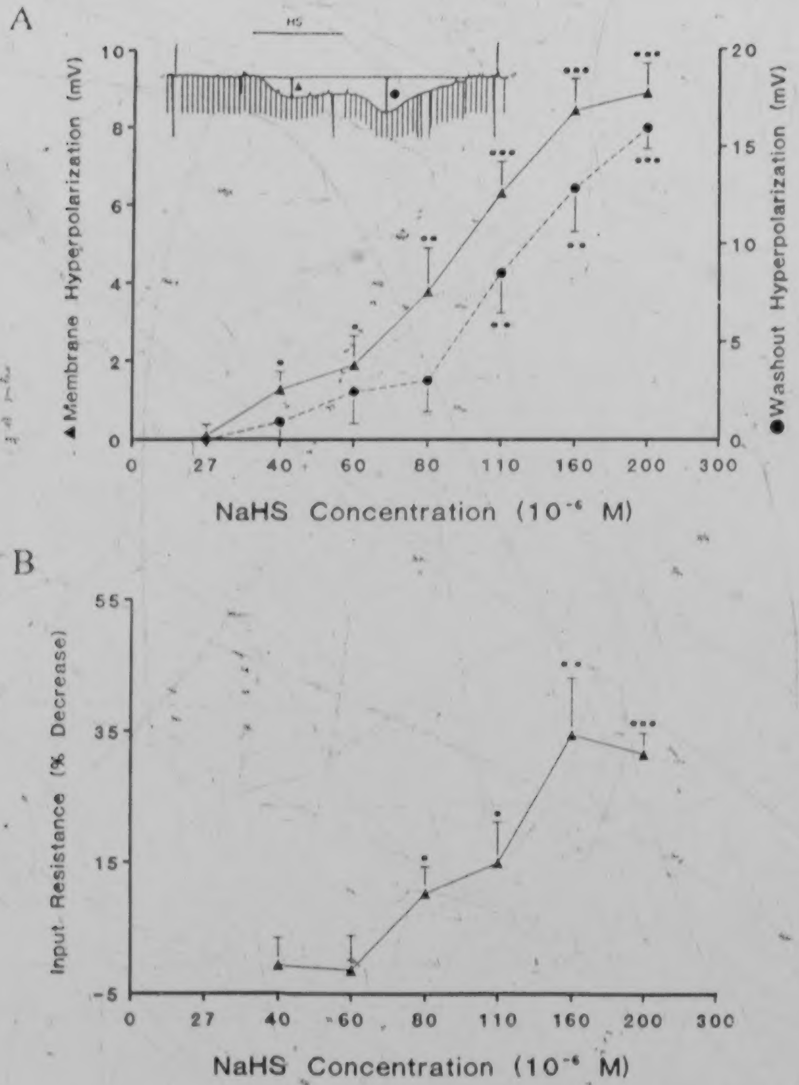


Fig. 8 Concentration-response relationships for HS$^-$ actions on CA1 neuron properties. A. Membrane hyperpolarization (EC$_{50}$ $\simeq$ 90 μM), and washout hyperpolarization (EC$_{50}$ $\simeq$ 100 μM) elicited by HS$^-$ in CA1 pyramidal cells. B. Decrease in input resistance in CA1 cells; EC$_{50}$ $\simeq$ 115 μM. (All values are means ± SEM; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to controls. The washout hyperpolarization was calculated as the maximum difference in membrane potential during the wash from the resting membrane potential preceding the NaHS application, see inset, Figure 8A.)

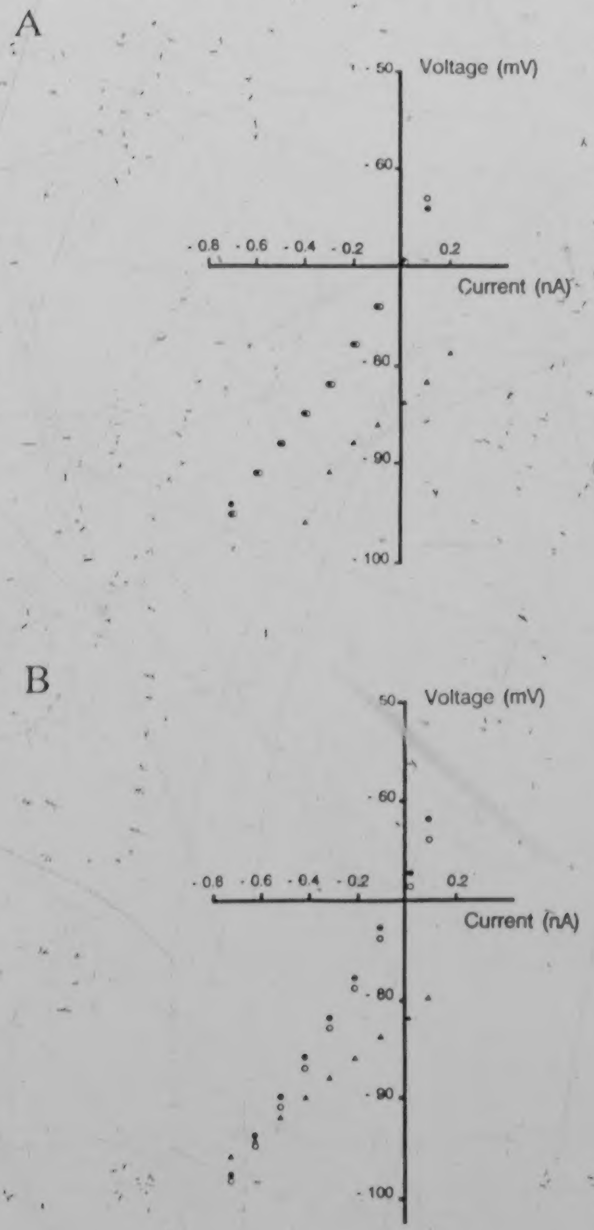


Fig. 9 Voltage-current (V-I) relationships, obtained in current clamp, of CA1 neurons. A. 80 μM HS^- significantly decreased the slope of the I-V relationship, indicating a reduction in neuronal input resistance. B. V-I relationship of another CA1 cell showing the reversal potential for the conductance change in response to application of 200 μM HS^-. (● control, ▲ HS^-, ○ wash)

2. "*Washout hyperpolarization*"

A further, significant, concentration-related membrane hyperpolarization ("washout hyperpolarization") was observed about 1.5-2 minutes after initiation of wash ($p < 0.02$, n=10) with normal ACSF after applications of higher concentrations of NaHS (≥ 110 μM) regardless of the duration of NaHS applications (Figures 7 and 8A; EC$_{50}$$\simeq$100 μM). This washout hyperpolarization was calculated as the maximum difference in membrane potential observed during the wash from the resting membrane potential preceding the NaHS application (see inset, Figure 8A).

3. *Effects of NaHS on synaptic transmission*

NaHS also caused a reversible reduction in the orthodromically-evoked intracellularly recorded EPSP and extracellularly recorded PS amplitude (Figure 7B and 7C). These effects were also concentration dependent. EPSP amplitude was reduced by NaHS (≥ 60 μM) by a maximum of 81.4 ± 3.2% of control (at 200 μM, n=15; EC$_{50}$≥ 80 μM, see Figure 10). In some cells, some reversible decreases in EPSP amplitude were observed even though there were no detectable changes in membrane potential or input resistance. This suggested that a distant dendritic or presynaptic site of action may be responsible for some of the HS$^-$-mediated effects (see below). PS amplitudes were significantly inhibited by NaHS (≥ 27 μM) by up to 100% of control (NaHS 110 μM, n=5; Figure 10). The concentration-response relationship for NaHS inhibition of PS amplitude was steeper than for its inhibition of EPSP amplitude (Figure 10).

Examination of the stimulus-response relationship of EPSP amplitude (Figure 11A) demonstrated that the maximum EPSP amplitude was more strongly inhibited by HS^- than were EPSP amplitudes elicited from stimuli near threshold. Because of the variability in threshold and the range of stimulus voltages applied in the different preparations, this relationship was only qualitatively assessed, and when done, the relationship was shown to be similar in every case. Likewise, the maximum PS amplitude in response to a strong stimulus was more greatly inhibited than were PS amplitudes elicited from stimuli near threshold (Figure 11B). Furthermore, the PS was more sensitive to the actions of HS^- than was the intracellularly recorded EPSP as can be seen by comparing Figures 11A and 11B.

Because there was some evidence to suggest that HS^- was more potent in depressing PS amplitude than EPSP amplitude, and because both the EPSP and PS reflect somatic events, experiments that would enable us to compare more directly the effects of HS^- on dendritic and somatic potentials (e.g. PS) were performed using the technique of Colmers et al. (1987). Thus, in some experiments, a second extracellular electrode was inserted into the corresponding apical dendritic region of CA1 to record the extracellular field EPSP. In these experiments, application of HS^- (60 μM) caused a depression in the amplitudes of

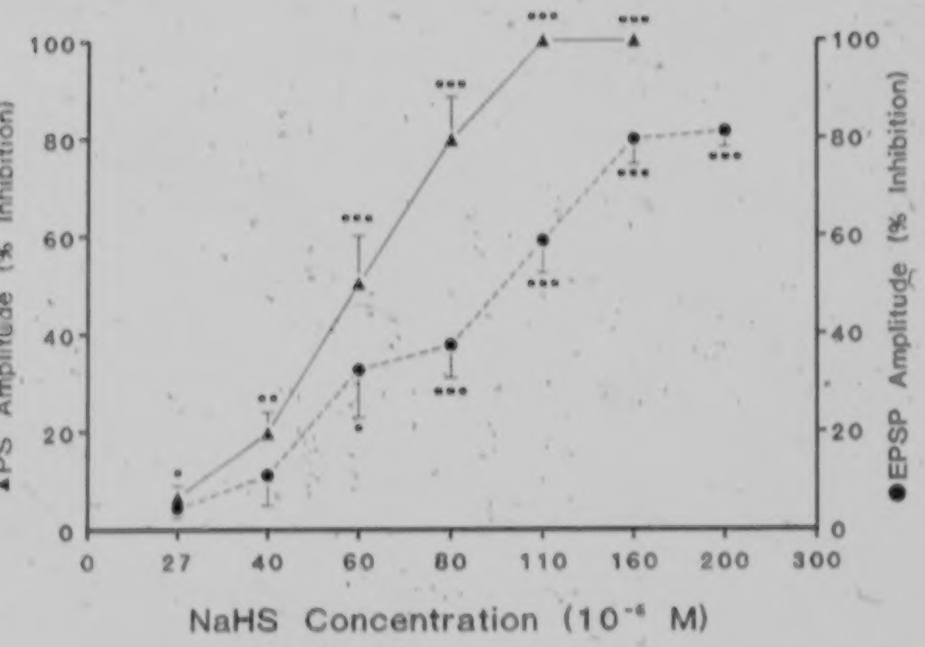


Fig. 10 Concentration-response relationships for HS^- actions on the orthodromically-evoked intracellularly recorded excitatory postsynaptic potential (EPSP) and extracellularly recorded population spike (PS) amplitude in area CA1. Inhibition of EPSP amplitude ($EC_{50} \cong 80\ \mu M$), and PS amplitude ($EC_{50} \cong 60\ \mu M$). All values are means ± SEM; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to controls (from immediately preceding responses in HS^--free ACSF.)

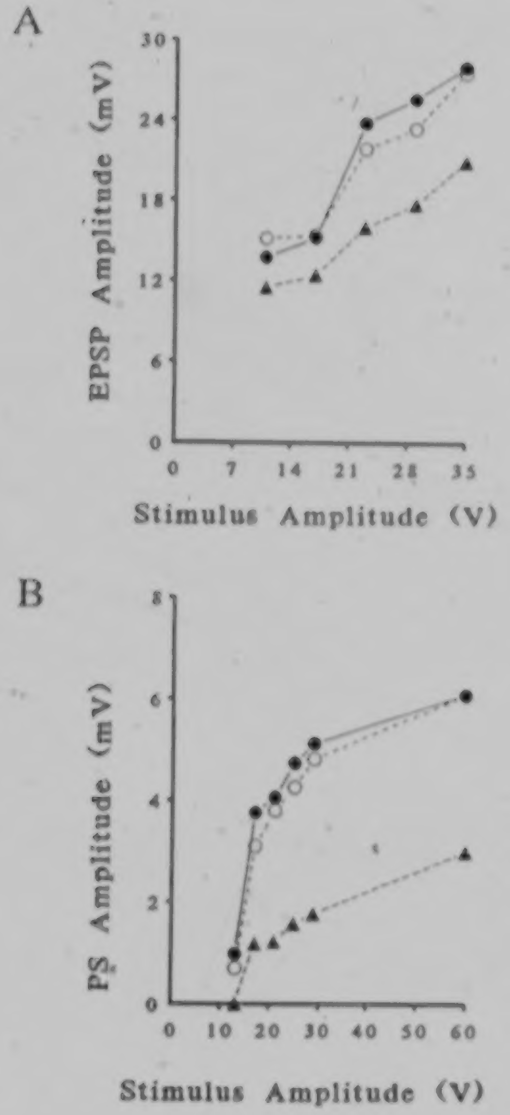


Fig. 11 Effects of 80 μM HS^- on the stimulus-response relationships of (A) the intracellularly recorded EPSP, and (B) the extracellularly recorded PS in area CA1 of rat hippocampus. Both EPSP's and PS's were elicited by stimulation of the *stratum radiatum* at the voltages indicated. Data for A and B were obtained from different experiments; in B, data shown were obtained from PS recorded in Figure 7C. (● control; ▲ 80 μM HS^-; ○ wash 10 min.)

the field EPSPs similar to that of the intracellularly recorded EPSPs (101.4 ± 12.8 %; n=4). Furthermore, in addition to reducing the amplitudes both of the PS and field EPSP, HS^- altered the relationship (as shown by the decreased slope) from that seen in control (see Figure 12). These results suggested that HS^- may have affected the cable properties of the postsynaptic cells and/or decreased the excitability of these cells by shifting the membrane potential away from action potential threshold.

4. Effects of NaHS on active membrane properties

When the membrane potential started to hyperpolarize as a result of HS^- application, if spontaneous spike activity occurred, it was markedly depressed, but somatic action potentials could still be elicited by applying depolarizing pulses of sufficient amplitude in all cells. When 80 μM HS^- was applied to 6 cells, no significant changes in action potential amplitude (98.5 ± 2.6 %), and action potential duration (99.6 ± 2.7 %) were observed (see Figure 13). Furthermore, the threshold voltage for action potential generation was unaffected by 80 μM HS^- (101.4 ± 1.0 %). Lastly, the long lasting or slow afterhyperpolarization (AHP), which follows current-induced repetitive firing in CA1 neurons (Hotson and Prince, 1980), was reduced in 2 out of 3 neurons (by 20.9 ± 12.3 %) by 80 μM HS. In the third neuron, the slow AHP was unaffected by 80 μM HS^-.

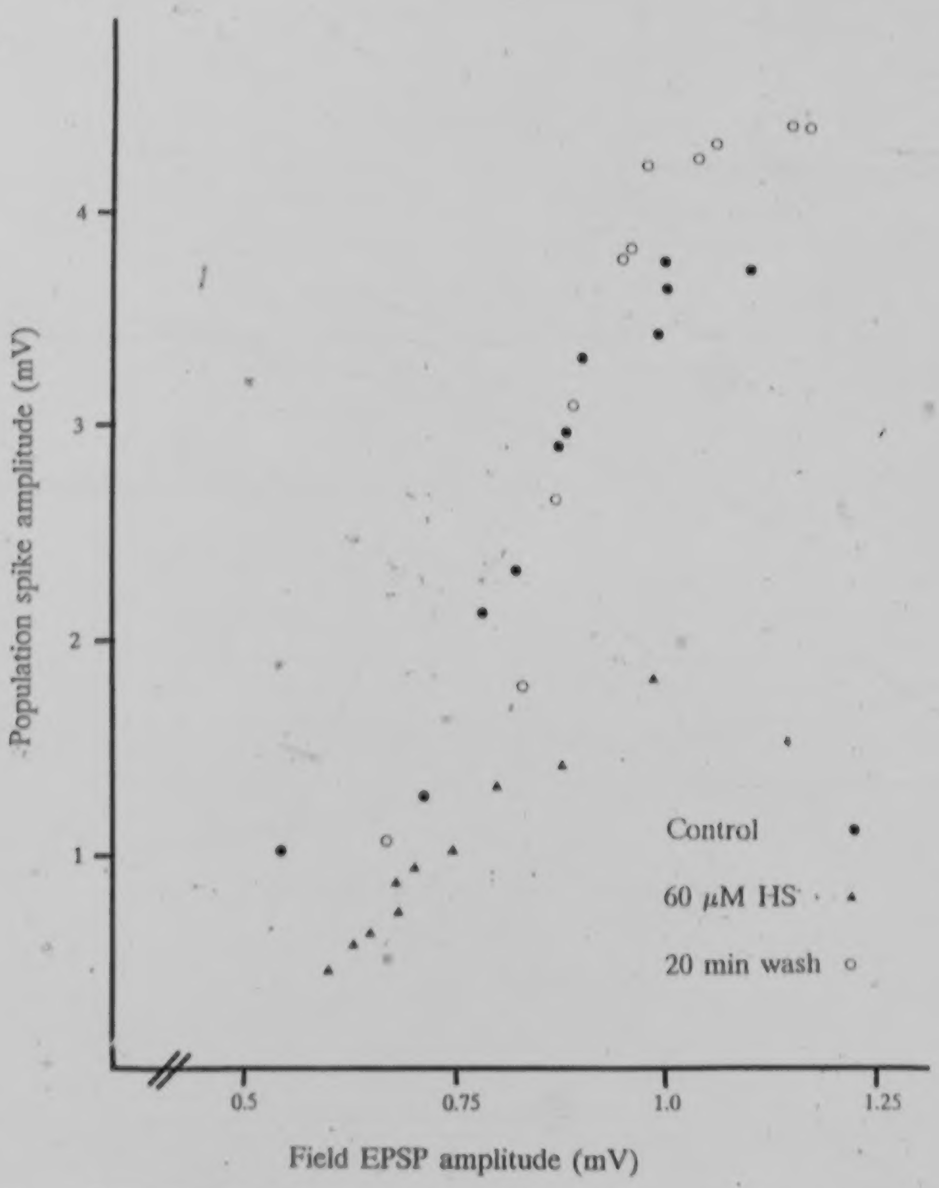


Fig. 12 Relationship between the amplitudes of the population spike and field EPSP recorded extracellularly from the cell body and apical dendritic regions respectively, of area CA1. Application of 60 μM HS⁻ (▲), not only caused a reduction in the amplitudes both of the EPSP and population spike, but also altered the relationship from that seen in control (●), 20 min wash (○).

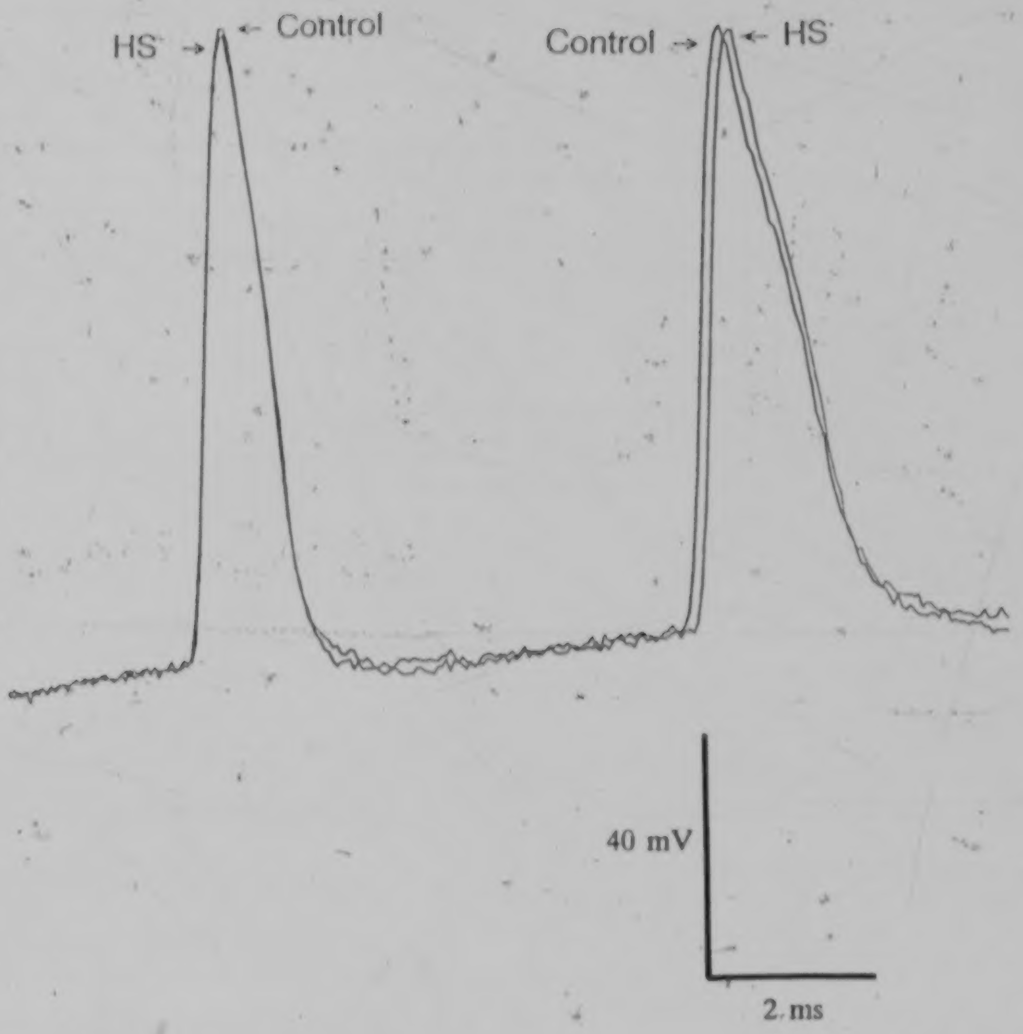


Fig. 13 Effects of HS^- on action potentials of a CA1 pyramidal cell. Membrane potential of the neuron was -64 mV. Action potentials elicited in control and 80 μM HS^- are superimposed. HS^- had no significant effect on CA1 cell action potential amplitude or duration.

B. Mechanism(s) of Action of NaHS

In all experiments involving extracellular applications of the various reagents, a control response to application of either 160 or 200 μM NaHS was obtained first. These concentrations of HS^- were chosen because their effects were pronounced and reproducible. After a recovery period of about 15-20 minutes, the drug was delivered to the preparation for about 6-8 minutes until a stable response was obtained. The same concentrations of HS^- were then applied in the presence of the drug for the same duration as that of control. After completion of the HS^- application, the perfusate was switched back to the drug-containing medium to obtain recovery data before returning to control ACSF. During experiments in which drugs were initially dissolved in absolute ethanol, ethanol controls ($\simeq$50 mM) were established using the same concentrations of ethanol as that employed during the drug applications. Prior to all ethanol applications, the final solution was bubbled vigorously and continuously with 95 % O_2, 5 % CO_2 for at least 5 minutes to allow some of the volatile ethanol to escape into the atmosphere.

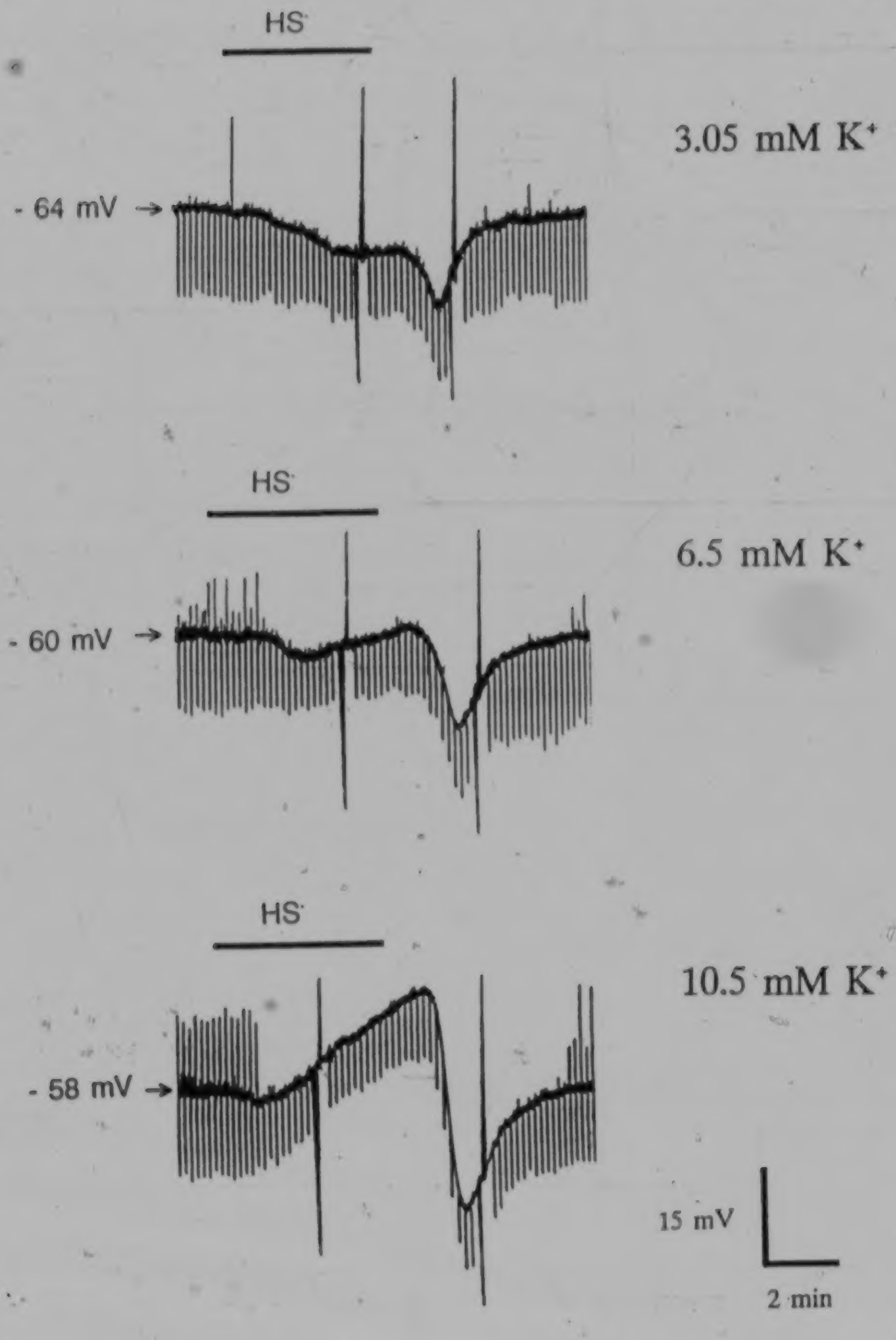
1. Experiments to determine the nature of the ionic conductance(s) altered by HS^-

Using intracellular recording, several experiments were performed to investigate the mechanism(s) underlying the initial hyperpolarization and its associated change in input resistance. This involved a series of protocols to determine which ionic conductance(s) may be altered by HS^-.

To determine if the HS$^-$-induced effects were mediated by changes in Cl$^-$ conductances, CA1 neurons were impaled with electrodes filled with 2 M KCl which would shift the Cl$^-$ reversal potential to a more positive voltage (Newberry and Nicoll, 1984a and b). Applications of 200 μM HS$^-$ still resulted in a pronounced hyperpolarization (-8.0 ± 1.1 mV) which was accompanied by a large decrease in input resistance (31.6 ± 5.6 %). This was not significantly different from that seen in neurons impaled with potassium acetate (KAc)-filled electrodes ($p > 0.5$ and $p > 0.9$ for membrane hyperpolarization and changes in input resistance, respectively). The sulfide-induced washout hyperpolarization was also unchanged in these experiments (-15.5 ± 1.6 mV; $p > 0.7$; n=4).

Since the results of this experiment suggest that the HS$^-$-mediated hyperpolarization was not due to an increase in Cl$^-$ conductance, experiments were performed to determine whether or not the effects caused by HS$^-$ were due to the activation of a potassium conductance. For example, in some experiments, the reversal potential for K$^+$ was shifted in a depolarizing direction by elevating the extracellular potassium concentration. In these experiments, elevation of extracellular potassium concentration to 6.5 and 10.5 mM led to a suppression of the hyperpolarization in response to application of 200 μM HS$^-$, but the washout response was not affected (Figure 14). However, during the HS$^-$ applications in these high-potassium-containing media, a depolarization, which commenced immediately after the initial

Fig. 14 HS$^-$-induced hyperpolarization and washout hyperpolarization responses: effects of elevation of extracellular potassium concentration. Chart records of membrane potential of a CA1 pyramidal neuron; downward deflections indicate passage of hyperpolarizing current through the electrode. Lower records were obtained about 30 min after the termination of the preceding record; application of high K^+-containing media commenced 6-8 min before, and continued until 3-5 min after HS$^-$ (200 μM) application. Elevation of extracellular K^+ concentrations to 6.5 and 10.5 mM led to a suppression in the HS$^-$-induced hyperpolarizations while the washout hyperpolarizations were not attenuated. Furthermore, a depolarization often followed the initial hyperpolarization during HS$^-$ application in these high-K^+-containing media. The amplitude of this depolarization caused by HS$^-$ increased with increasing concentrations of extracellular K^+.


HS⁻
3.05 mM K⁺
- 64 mV →
HS⁻
6.5 mM K⁺
- 60 mV →
HS⁻
10.5 mM K⁺
- 58 mV →
15 mV
2 min

hyperpolarization, was observed. The amplitude of this depolarization caused by HS^- increased with increasing concentrations in extracellular potassium and reached up to a maximum of 20 mV (200 uM HS^- in 10.5 mM K^+; measured from the resting membrane potential preceding the HS^- application). The maximum hyperpolarization in these high-potassium-containing media, (-4.7 ± 0.8 mV, and -2.5 ± 0.7 mV, respectively) was significantly smaller ($p < 0.01$) than those observed in the same neurons in control medium (3.05 mM K^+, -10.3 ± 1.2 mV; Figure 14). However, applications of 6.5 and 10.5 mM K^+ did not alter the HS^--mediated changes in neuronal input resistances (e.g. 87.6 ± 9.3 % and 96.5 ± 5.0 % of controls, respectively). Interestingly, the washout hyperpolarizations in both 6.5 mM K^+ (-12.8 ± 2.5 mV, n=6; $p > 0.7$) and 10.5 mM K^+ (-13.0 ± 3.4 mV, n=4; $p > 0.6$) after application of 200 μM NaHS did not differ significantly from control (-17.7 ± 0.7 mV, n=6; Figure 14).

In order to delineate the mechanism(s) underlying the change in ionic conductance caused by HS^-, in some experiments, single microelectrode voltage clamp was performed in cells impaled with electrodes filled with KAc (2 M). Slow voltage command ramps were applied and the cells were slowly depolarized from -120 mV to -40 mV. The steady-state current-voltage (I-V) relationships, obtained in the absence (control) and presence of 200 μM HS^-, were not linear (Figure 15). The slope of the steady state (I-V) lines increased progressively as the membrane potential was made more negative (probably due to some inward

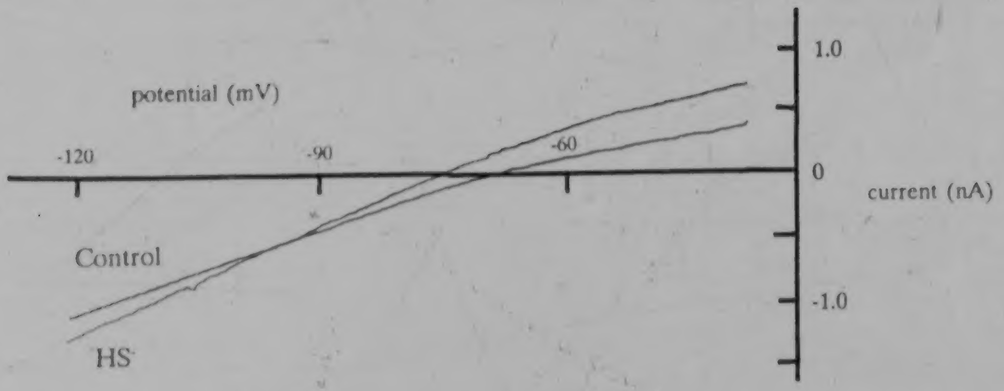


Fig. 15 Current-voltage (I-V) relationships of a CA1 pyramidal cell obtained by slow ramp depolarization from -120 to -40 mV in control and in the presence of HS⁻ (200 μM). When the neuron was clamped at the resting membrane potential, HS⁻ induced an outward current, reflecting the hyperpolarization seen during current clamp mode. The point of intersection of the control and HS⁻ I-V relationships, which represents the reversal potential for the HS⁻-mediated conductance increase, was about -95 mV. At membrane potentials above the reversal potential, the HS⁻-induced conductance increase was due to an outward current, whereas at membrane potentials below the reversal potentials below the reversal potential, it was due to an inward current.

rectification; Krnjević and Leblond, 1989a; Leblond and Krnjević, 1989; Williams et al., 1988). In neurons clamped at the resting membrane potential, HS^- induced an outward current, reflecting the hyperpolarization seen in current clamp mode. The overall slope of the I-V relationship between -120 and -40 mV increased during the HS^- application, indicating an increased conductance of the cell. Lastly, the point of intersection of the control and HS^- I-V relationships, which represents the reversal potential for the HS^--mediated conductance increase, was about -95 mV. At membrane potentials above the reversal potential, the HS^--induced conductance increase was due to an outward current, whereas at membrane potentials below the reversal potential, it was due to an inward current.

Although the reversal potential obtained from the voltage clamp experiment was slightly more positive than the calculated potassium equilibrium potential (E_K) for these cells ($E_K \simeq$ -105 mV; Segal and Barker, 1984; Hille, 1984), this underestimation could be attributed to problems inherent in the experimental technique, such as inadequate space clamp. The reversal potential obtained from V-I plots during current clamp (99.8 ± 5.0 mV; see above) was also more positive than E_K; this was likely due to progressive inward rectification seen during hyperpolarizing pulses (Krnjević and Leblond, 1989a).

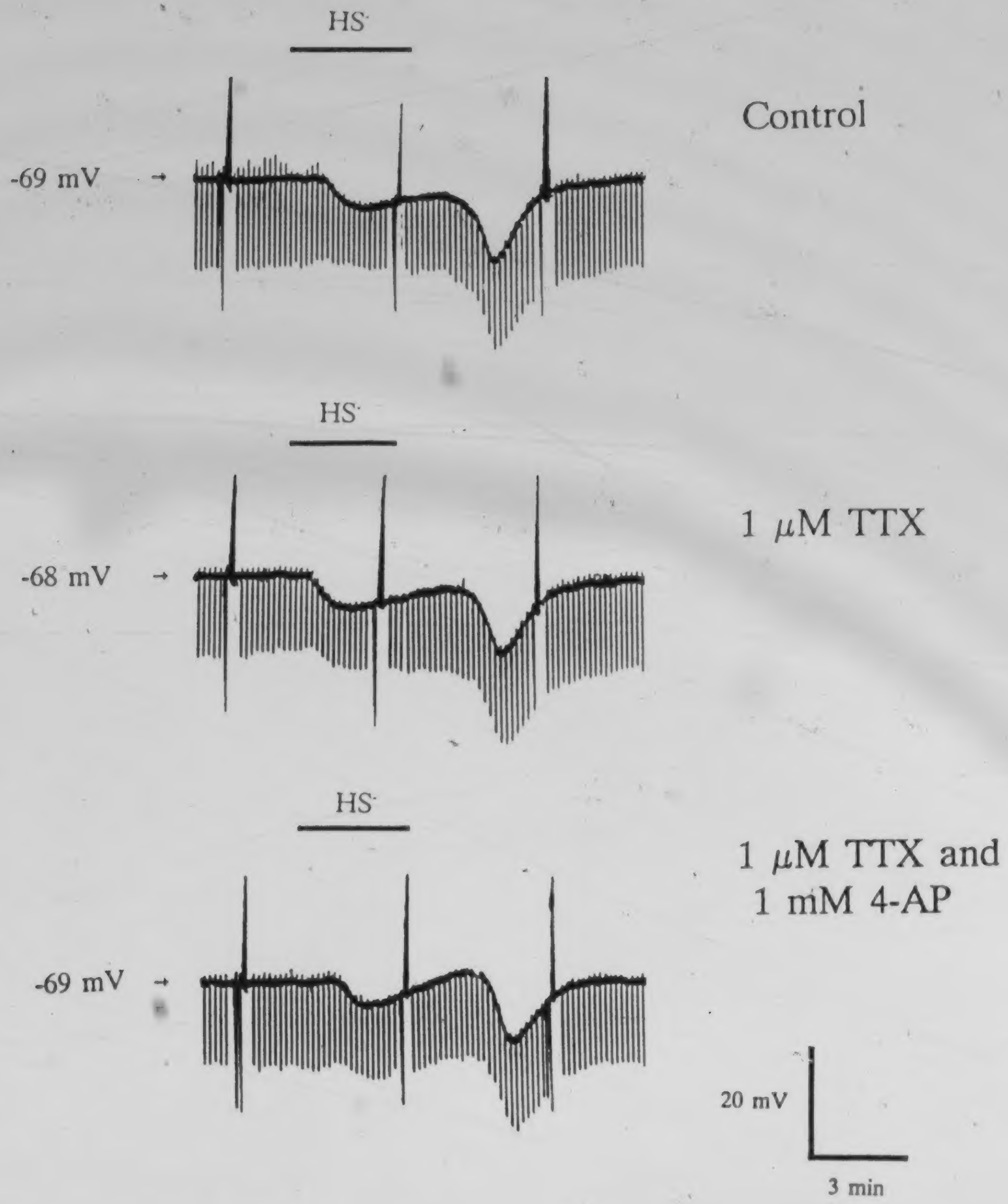
2. *Experiments to determine the nature of the HS⁻-induced K⁺-conductance*

Once there was some evidence to suggest that the initial HS⁻-induced hyperpolarization seen in CA1 pyramidal cells was most likely due to an increase in membrane conductance to K^+ ions, I performed experiments to determine which K^+-conductance(s) may be contributing to these HS⁻-mediated effects, using antagonists of several different K^+-channels. In all experiments the HS⁻-induced effects seen in the presence of the various K^+-channel antagonists were compared to the effects caused by the same concentration of HS⁻ applied alone (controls) in the same neurons. The only exceptions included experiments in which cells were impaled with microelectrodes filled with BAPTA or Mg-ATP (in potassium acetate; KAc) or CsCl:CsAc (4:1); in these experiments, the HS⁻-induced effects under these conditions were compared to those effects caused by the same concentration of HS⁻ seen in cells impaled with microelectrodes filled with KAc.

A fast, transient, outward K^+-current ("A" current; I_A) has been observed in many excitable cells, including hippocampal neurons *in vitro* (Connor and Stevens, 1971; Segal and Barker, 1984; Rudy, 1988). In order to test the hypothesis that the voltage-dependent I_A may have been contributing to the HS⁻-induced effects, the antagonist, 4-aminopyridine (4-AP; 1 mM) was applied extracellularly to block these channels (Segal and Barker, 1984). These experiments were conducted in the presence of extracellular tetrodotoxin (TTX; 1 μM) to prevent the spontaneous release of transmitter and epileptiform bursting caused by 4-AP due to its action on presynaptic terminals (Thesleff, 1980; Buckle and Haas, 1984). In these experiments, application of 4-AP and TTX did not alter the membrane potential or the input resistance near the resting potential of the cells. During HS$^-$ applications, no reductions in the initial hyperpolarization (92.8 ± 21.4 % of controls in the same neurons, n=3; Figure 16) or the changes in input resistance (76.3 ± 17.1 %) were observed. Furthermore, the HS$^-$-induced washout hyperpolarization was not affected by these concentrations of TTX and 4-AP as compared to controls in the same neurons (96.7 ± 16.7 %).

Extracellular tetraethylammonium (TEA) has been reported to block many K$^+$-currents including voltage-dependent "delayed rectifiers" and some Ca^{2+}-activated K$^+$-channels in many excitable cells including CA1 pyramidal neurons (Rudy, 1988; Segal and Barker, 1984). In CA1 cells, I_A has been reported to be relatively insensitive to TEA (Segal and Barker, 1984). In our experiments, bath application of 20 and 50 mM TEA, in the presence of 1 μM TTX extracellularly (to eliminate spontaneous activity; Schwartzkroin and Prince, 1980), raised the apparent neuronal input resistance by about 50 and 70 % (respectively), but membrane potential was relatively unchanged. However, application of 50 mM TEA led to a significant suppression of the hyperpolarization induced by 200 μM HS$^-$: 57.9 ± 14.8 % ($p < 0.05$, n=4; Figure 17), while the change in input resistance caused by 200 μM HS$^-$ was reduced to 54.2 ± 15.0 % of that seen in normal ACSF control. The application of 20 mM TEA had no significant effects on the hyperpolarization or the change in input resistance caused by HS$^-$. Moreover, the

Fig. 16 HS$^-$-induced effects are unaffected by extracellular TTX and 4-AP. All chart records of membrane potential were obtained from the same CA1 cell of rat hippocampal slice; downward deflections indicate passage of hyperpolarizing current through the electrode. Lower records were obtained about 25 min after the termination of the preceding record; all drug applications commenced 6-8 min before, and continued until 3-5 min after HS$^-$ (200 μM) application (time indicated by horizontal bar) except TTX which was continuously applied throughout once application was initiated. Application of TTX (1 μM; alone; to block action potential-mediated transmitter release) and in the presence of the potassium channel blocker, 4-AP (1 mM) does not lead to any significant changes in the HS$^-$-mediated effects.


HS⁻
Control
-69 mV →
HS⁻
1 μM TTX
-68 mV →
HS⁻
1 μM TTX and
1 mM 4-AP
-69 mV →
20 mV
3 min

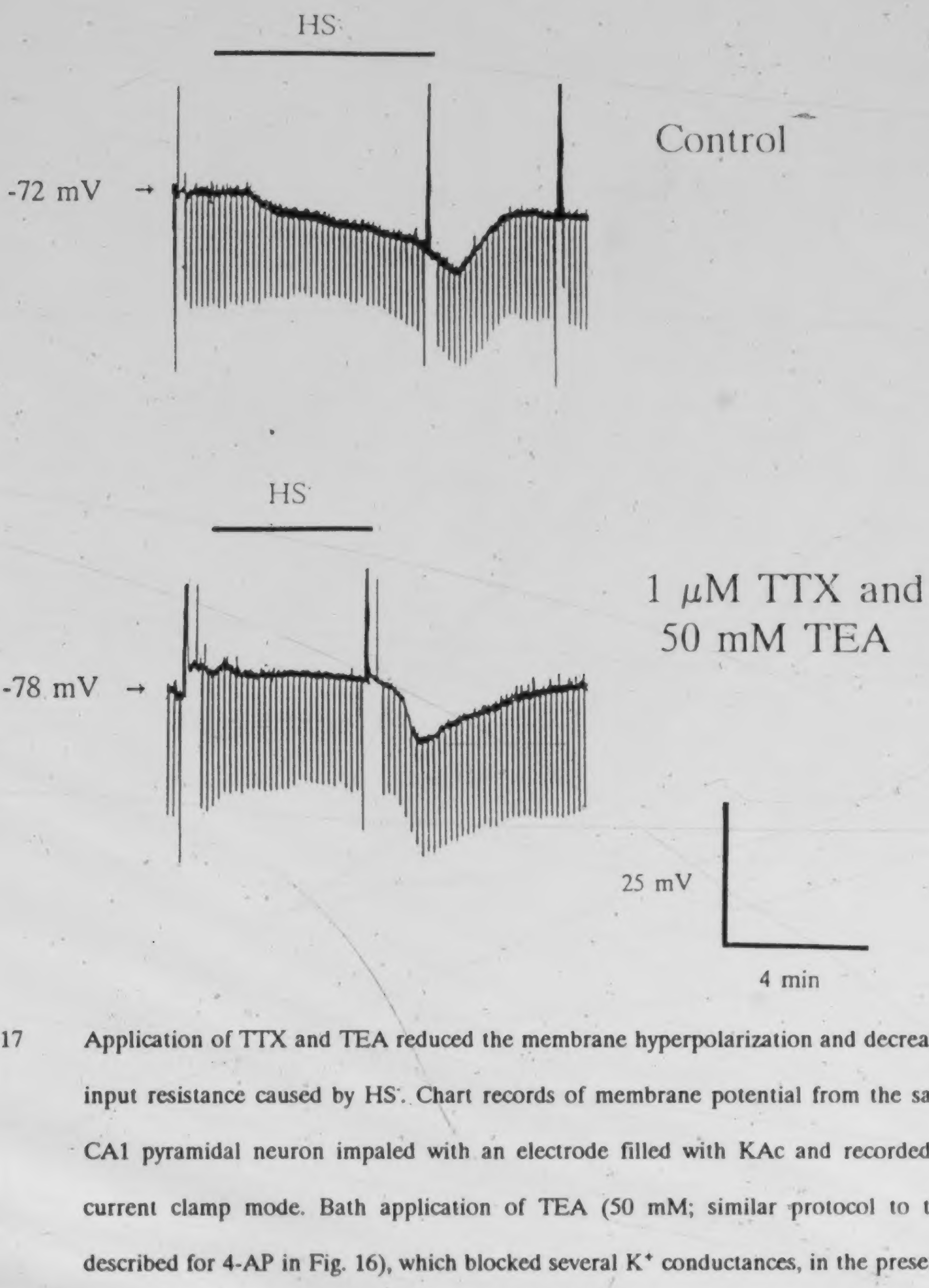


Fig. 17 Application of TTX and TEA reduced the membrane hyperpolarization and decreased input resistance caused by HS^-. Chart records of membrane potential from the same CA1 pyramidal neuron impaled with an electrode filled with KAc and recorded in current clamp mode. Bath application of TEA (50 mM; similar protocol to that described for 4-AP in Fig. 16), which blocked several K^+ conductances, in the presence of TTX (1 μM), raised the apparent neuronal input resistance while membrane potential was unchanged. Furthermore, TTX and TEA led to a significant reduction in the HS^--induced hyperpolarization and change in input resistance; however, the washout hyperpolarization caused by HS^- was not affected.

washout hyperpolarization caused by HS^- application was not affected by 50 mM TEA (97.2 ± 29.0 %).

Inwardly-rectifying (or anomalously rectifying) K^+-conductances, which increase in response to membrane hyperpolarization, have been reported in many excitable cells including hippocampal CA1 pyramidal cells (Hille, 1984; Rudy, 1988; Segal and Barker, 1984). In order to determine whether or not the activation of inwardly-rectifying K^+-conductances may have contributed to the HS^--mediated effects, extracellular CsCl (Cs^+, 2 mM), an antagonist of these conductances (Rudy, 1988; Segal and Barker, 1984), was applied in some experiments. Application of extracellular Cs^+ raised the neuronal input resistance by about 30 % while no significant changes in membrane potential were observed. However, the hyperpolarization induced by 200 μM HS^- was significantly reduced in the presence of extracellular Cs^+ (by 66.4 ± 11.8 %; $p < 0.01$, n=6; Figure 18), although the reduction in input resistance caused by HS^- was unaltered (78.0 ± 15.0 %) compared with that seen in controls in the same neurons. Interestingly, the HS^--induced washout hyperpolarization was significantly reduced in the presence of extracellular Cs^+ to 60.8 ± 11.9 % ($p < 0.05$) as compared to controls.

Since intracellularly applied Cs^+ has been shown to block several outward K^+-currents in a variety of excitable cells (Johnston et al., 1980; Puil and Werman, 1981), CA1 cells (n=7) were impaled with microelectrodes filled with CsCl:CsAc (2 M). In all cells, characteristically prolonged action potentials were observed upon synaptic stimulation or subsequent to direct depolarizations (c.f. Puil and Werman, 1981). Hyperpolarizing current was applied to prevent these neurons

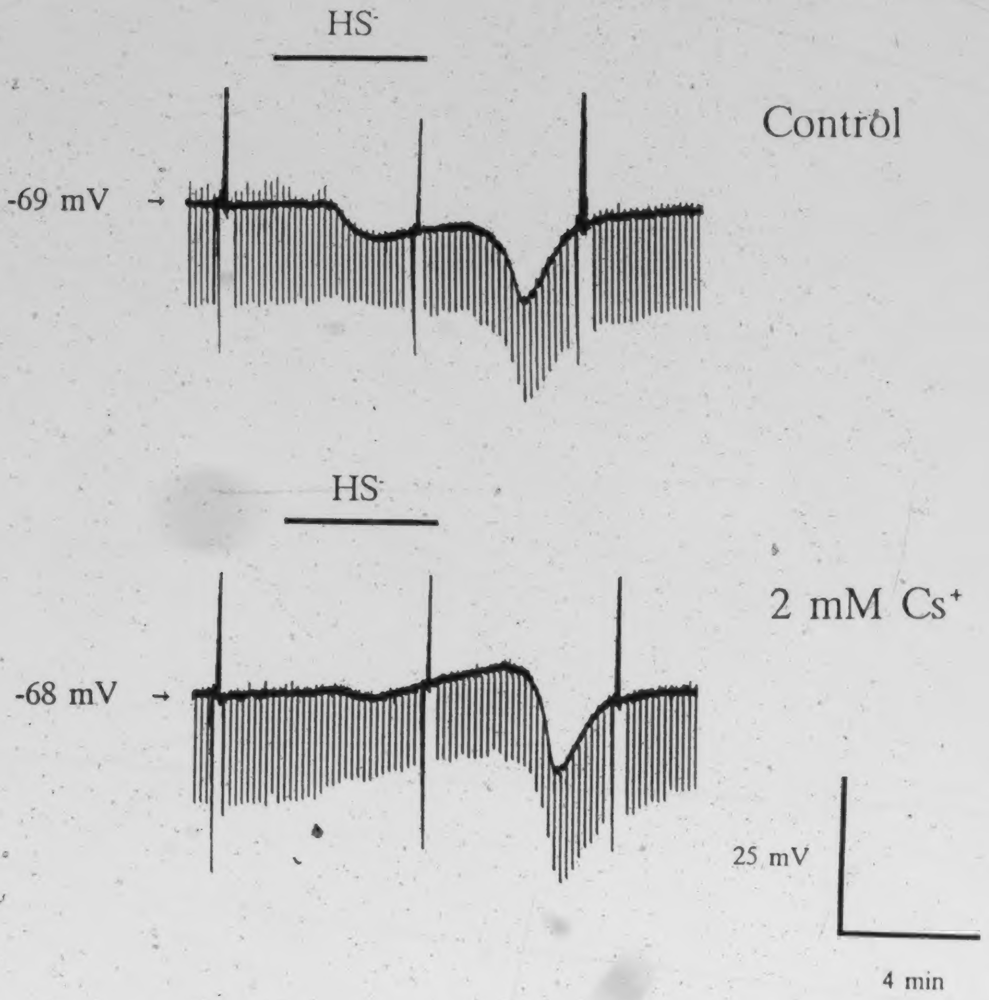


Fig. 18 Effects of extracellular Cs^+ on the effects caused by HS^-. Chart records of membrane potential from the same CA1 neuron; downward deflections indicate passage of hyperpolarizing current through the electrode. Bath application of Cs^+, which blocked inwardly-rectifying K^+ currents, raised neuronal input resistance, but membrane potential was unchanged. Cs^+ reduced the hyperpolarization induced by HS^- (200 μM), but the reduction in input resistance caused by HS^- was unaltered. The HS^--mediated washout hyperpolarization was also reduced by Cs^+.

from depolarizing spontaneously. The input resistances of these cells were relatively high, for example, 27.0 ± 17.4 % (n=7) higher than the input resistances observed in cells impaled with electrodes filled with KAc. In all 7 neurons, no initial hyperpolarizations were observed in response to applications of either 160 or 200 μM HS^-. In 5 neurons, application of 160 μM HS^- led to a depolarization (17.4 ± 7.9 mV). In 4 cells, a marked depolarization (29.5 ± 3.1 mV; $p < 0.01$; Figure 19) was observed during applications of 200 μM HS^-. The time of onset for the depolarization seen in these experiments was similar to the time required for commencement of the HS^--mediated hyperpolarization observed when cells were impaled with KAc filled electrodes. Because of the HS^--induced depolarization seen in the presence of intracellular Cs^+, the cells were hyperpolarized with negative current to return their membrane potentials to those observed before the HS^- application for measurements of input resistances. No significant changes in neuronal input resistances were observed (the resistances actually increased slightly: 6.4 ± 10.0 % and 4.1 ± 6.0 % for applications of 160 and 200μM HS^-, respectively). However when 160 μM HS^- was applied in these experiments, the washout hyperpolarization was significantly smaller (-3.4 ± 1.8 mV, $p < 0.01$) than that seen in neurons recorded with electrodes filled with KAc. On the other hand, the washout hyperpolarization caused by 200 μM HS^- (-8.8 ± 3.3 mV, $p > 0.1$) was not significantly diminished in these experiments (compared to KAc recordings). Although intracellular Cs^+ led to an inhibition and possibly a reversal of the HS^--induced hyperpolarization and its associated decrease

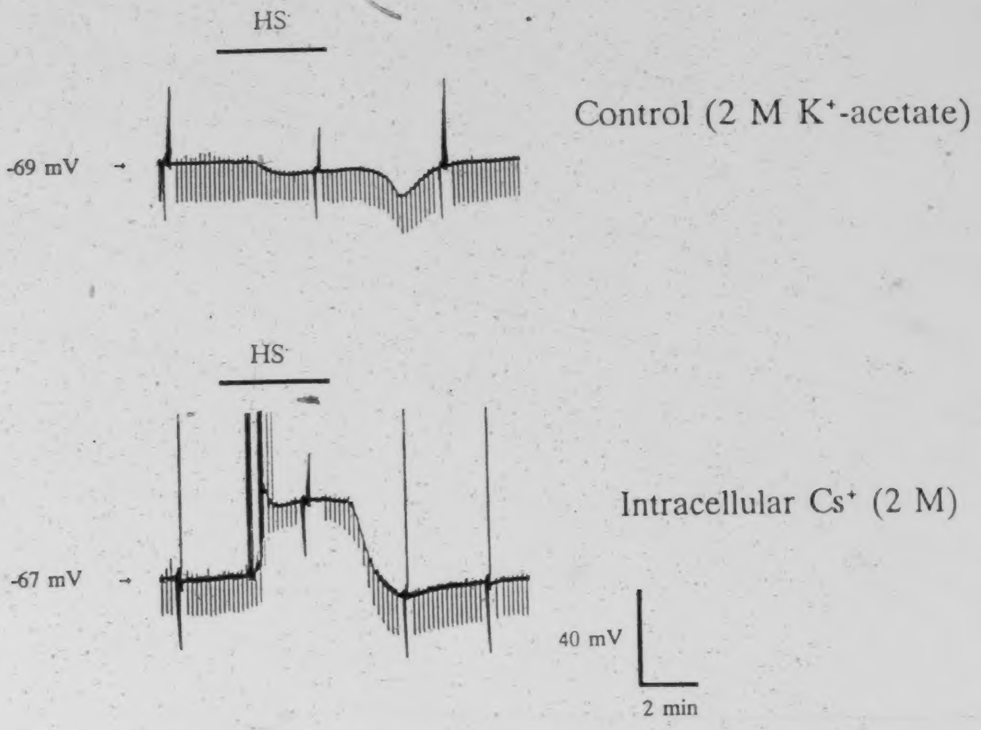


Fig. 19 Effects of intracellular Cs^+ on the HS^--mediated changes in membrane potential in CA1 neurons *in vitro*. Chart records of records of membrane potential; downward deflections indicate passage of hyperpolarizing current through the electrode. (Hyperpolarizing current (300 pA) was applied throughout to prevent the neuron from firing spontaneously.) Application of HS^- (200 μM), to a cell impaled with an electrode filled with KAc (2 M; A1), caused a hyperpolarization which was associated with a decreased input resistance; however, the same concentration of HS^-, applied to a cell impaled with an electrode filled with CsCl:CsAc (2 M; A2), caused a marked depolarization. Furthermore, the time of onset for both the hyperpolarization and depolarization observed in each experiment was similar. The HS^--induced washout hyperpolarization was slightly smaller in the presence of intracellular Cs^+ as compared to the control (KAc recording).

in input resistance, the amplitude of the intracellularly recorded EPSP's, evoked by *stratum radiatum* stimulation, were still attenuated to 31.1 ± 12.4 % ($p < 0.01$, $n=5$; 160 μM HS$^-$, Figure 20) and 24.2 ± 8.0 % ($p < 0.02$, $n=3$; 200 μM HS$^-$) of controls. These HS$^-$-mediated decreases in EPSP amplitude did not differ significantly from those observed using microelectrodes filled with KAc ($p > 0.5$ and $p > 0.6$ for 160 and 200 μM HS$^-$, respectively).

It was possible that H_2S may have caused a decrease in the availability of intracellular ATP, perhaps via inhibition of mitochondrial oxidative phosphorylation (Beauchamp et al., 1984), and this may have been responsible for the HS$^-$-mediated effects seen in these cells. For example, it has been reported that low levels of intracellular ATP activate ATP-sensitive potassium conductances (gK_{ATP}) in some cells (Cook and Hales, 1984). Therefore, in some experiments, CA1 pyramidal neurons were impaled with electrodes filled with 5 mM Mg-ATP in 2 M KAc to block gK_{ATP} (Cook and Hales, 1984). Intracellular ejections of Mg-ATP, via continuous application of hyperpolarizing current, did not significantly reduce the effects of application of 200 μM HS$^-$ including membrane hyperpolarization (117.0 ± 12.0 %, $n=8$ (4 cells); see Figure 21), decreases in input resistance (72.8 ± 13.8 %), or washout hyperpolarization (95.7 ± 4.1 %) compared to those of controls using electrodes filled with KAc (2 M) alone.

Activation of some K^+-currents have been reported to occur in response to rises in cytosolic Ca^{2+} and this leads to a Ca^{2+}-activated K^+-conductance (gK_{Ca2+}; Rudy, 1988). In CA1 pyramidal neurons, applications of intracellular BAPTA, a

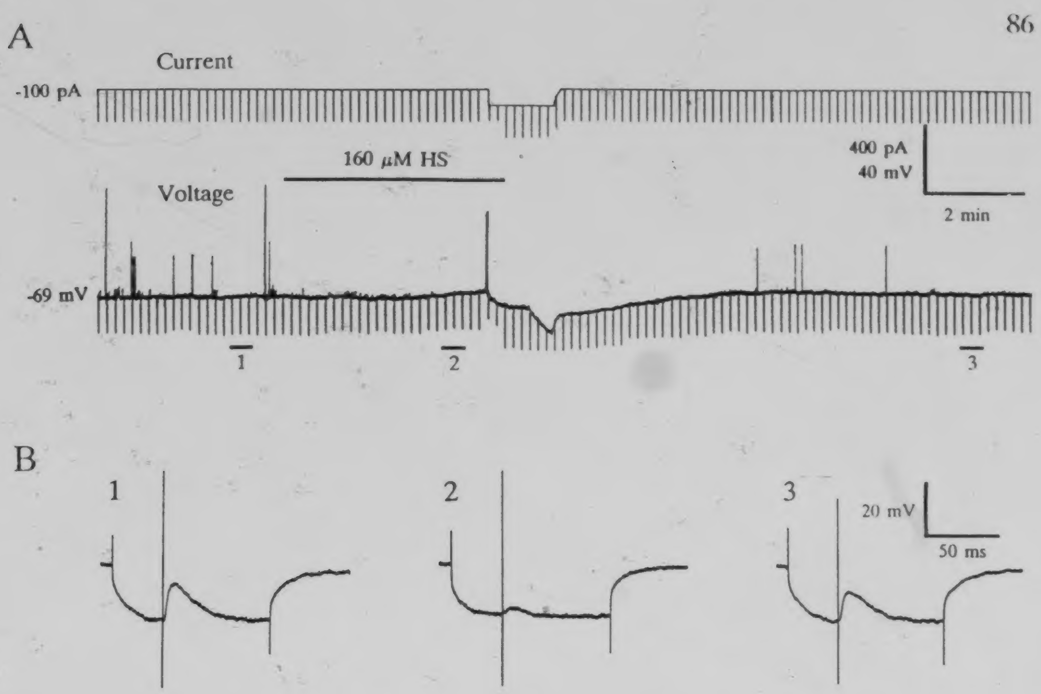


Fig. 20 Effects of intracellular Cs^+ on the effects caused by HS^- in a CA1 cell. A. Upper trace: intracellular current. Downward deflections indicate passage of hyperpolarizing current through the electrode. Lower trace: intracellular voltage. HS^- was bath-applied at 160 μM during time indicated by the horizontal bar. B. Digital oscilloscope traces at an expanded time scale of EPSP's evoked during brief hyperpolarizing pulses. A. Application of HS^- causes a small depolarization in the presence of intracellular Cs^+ (holding current was increased to prevent the cell from firing spontaneously). Furthermore, the HS^--induced decrease in input resistance normally seen when cells were impaled with electrodes filled with KAc is almost completely abolished in this experiment. B. Digital averages of 3 successive stimuli (labelled as 1, 2, and 3 in A). B1, orthodromically evoked EPSP in the absence of HS^-. B2, a large reduction in the evoked EPSP amplitude was observed during HS^- application. B3, EPSP amplitude recovered almost completely about 10 min after wash.

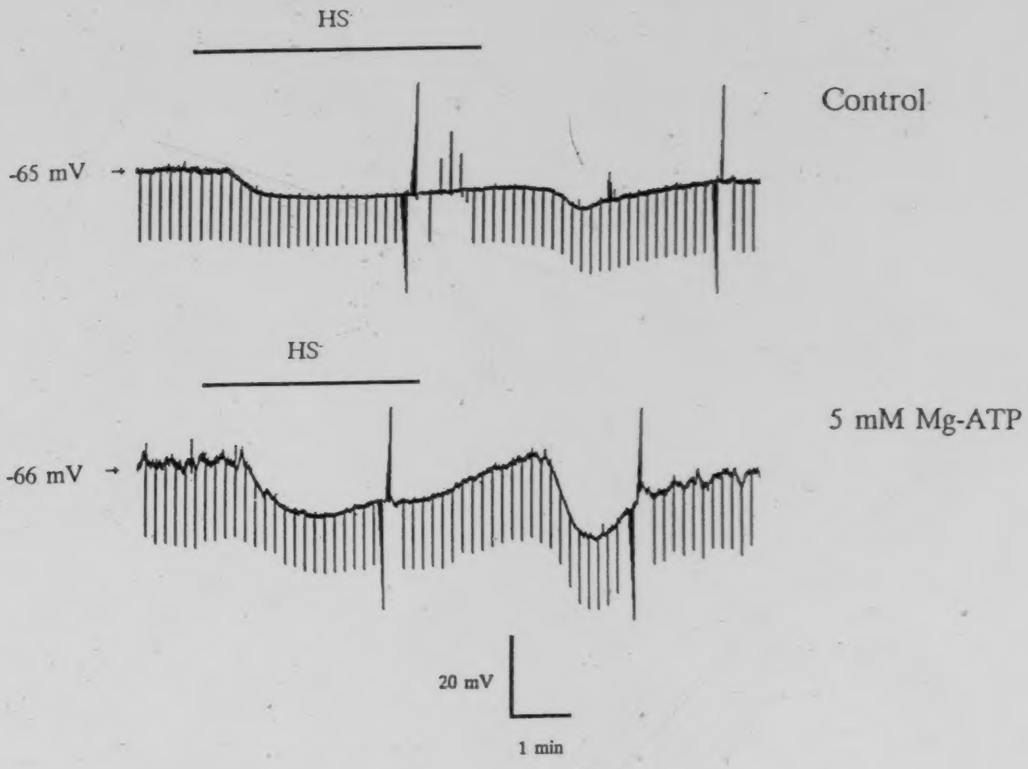


Fig. 21 Intracellular injections of Mg-ATP do not affect the HS^--mediated hyperpolarization and decreased input resistance seen in CA1 pyramidal cells. Chart records of membrane potential; downward deflections indicate passage of hyperpolarizing current through the electrode. Upper trace, hyperpolarization and decreased input resistance seen in a cell impaled with an electrode filled with KAc (2 M) occurred in response to application of HS^- (200 μM). Lower trace, the HS^--induced hyperpolarization and decreased input resistance is unaffected in another cell impaled with an electrode filled with Mg-ATP (5 mM) in KAc (2 M). Furthermore, the washout hyperpolarization was not affected by intracellular application of Mg-ATP as compared to controls (upper trace).

Ca^{2+} chelator, have been reported to block these conductances (Lancaster and Nicoll, 1987; Storm, 1987). In light of this, some neurons were impaled with electrodes filled with BAPTA (50 mM) in KAc (2 M). In 4 of the 5 cells, negative current (0.1 to 0.2 nA) was continuously applied to inject BAPTA into the cells. (In the fifth neuron, no current was applied.) Neurons impaled with these electrodes did not differ significantly from controls (i.e. cells impaled with electrodes filled with KAc (2 M) alone) in membrane potential (-66.8 ± 1.2 mV; $n=5$, $p > 0.6$) or input resistance (103.2 ± 5.4 %, $n=5$). Furthermore, BAPTA has been shown to block the fast afterhyperpolarization (AHP), which is mediated by $gK_{Ca^{2+}}$, and also to inhibit repolarization and broaden action potential spikes in CA1 cells (Lancaster and Adams, 1986; Lancaster and Nicoll, 1987; Storm, 1987). In these cells, action potential duration was significantly increased to 112.0 ± 3.2 % ($p < 0.02$; spike width measured at about one-third of full amplitude, c.f. Storm, 1987), while action potential amplitude was not significantly affected (100.3 ± 2.2 %), as compared to controls. Moreover, since BAPTA has also been reported to block the slow AHP which follows current-induced repetitive firing (Storm, 1987; Lancaster and Nicoll, 1987), the effects of intracellularly applied BAPTA on the slow AHP in these cells were examined. In 4 cells, BAPTA caused a significant reduction in the slow AHP (17.0 ± 3.8 %; $p < 0.05$). In these experiments, intracellular injections of BAPTA (in KAc) led to a significant suppression of both the hyperpolarization induced by 200 μM HS^- (by 45.9 ± 10.8 %; $p < 0.02$, $n=5$; Figure 22) and changes in neuronal input resistance (by 47.5 ± 12.3 %; $p < 0.02$)

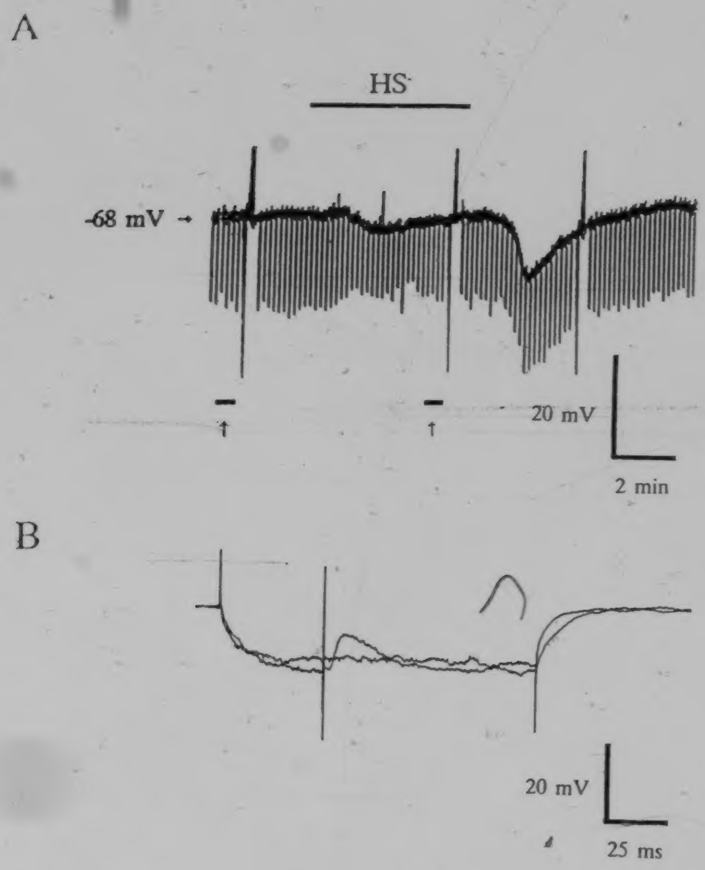


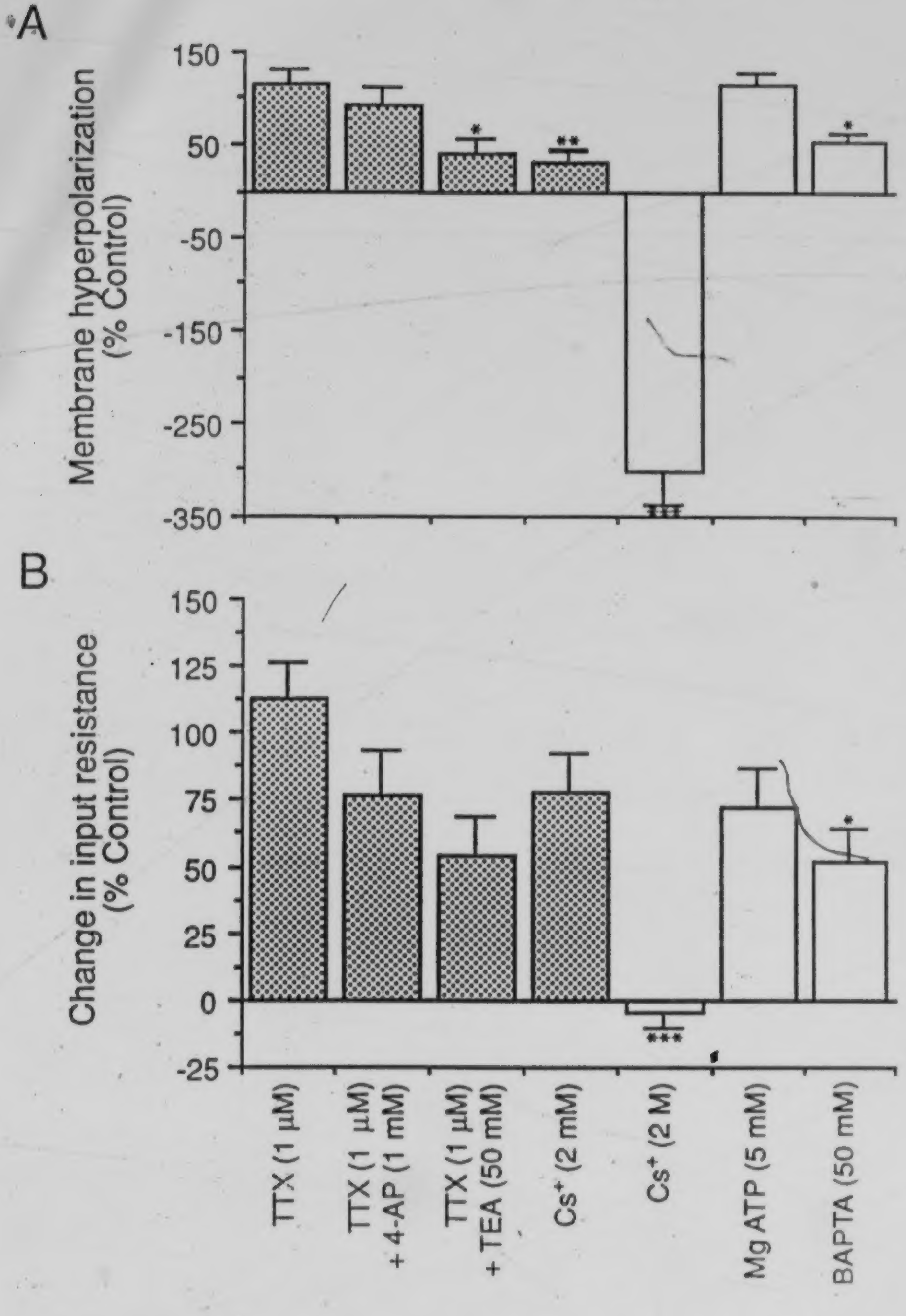
Fig. 22 Effects of intracellular injections of BAPTA on the HS^--mediated effects observed in CA1 neurons *in vitro*. A. Chart record of membrane potential of neuron impaled with electrode filled with BAPTA (50 mM) in KAc (2 M), and B digital oscilloscope traces at an expanded time scale of EPSP's evoked during brief, constant current hyperpolarizing pulses. A. Application of intracellular BAPTA leads to a significant reduction of both the hyperpolarization induced by HS^- (200 μM) and changes in input resistance as compared to that of control neurons impaled with electrodes filled with KAc (see Figure 7). The HS^--mediated washout hyperpolarization was unaffected by BAPTA. B. Digital averages of 3 successive stimuli (arrows in A); control and sulfide traces are superimposed. The orthodromically-evoked EPSP amplitude was still significantly attenuated by HS^-.

compared to those of control neurons impaled with electrodes filled with KAc. However, the HS$^-$-induced washout hyperpolarization was not significantly affected in BAPTA-filled cells as compared to controls (97.9 ± 9.4 %; $p < 0.02$). Interestingly, the amplitude of the intracellularly recorded EPSP's, evoked by *stratum radiatum* stimulation, were still significantly attenuated to 21.6 ± 3.0 % ($p < 0.001$; $n=5$) when HS$^-$ (200 μM) was applied in these experiments. These HS$^-$-induced decreases in EPSP amplitude did not differ significantly from those observed using microelectrodes filled with KAc ($p > 0.4$). A summary of the effects of the K$^+$-channel blockers, TTX, or intracellular injections of Mg-ATP or BAPTA on the effects caused by application of HS$^-$ (200 μM) on CA1 pyramidal cells is shown in Figures 23 and 24.

3. Experiments to determine mechanism(s) involved in HS$^-$-induced washout hyperpolarization

As mentioned above, in experiments in which pyramidal cells were impaled with microelectrodes filled with 2 M KCl, no significant reductions in the HS$^-$-mediated washout hyperpolarization were observed. Furthermore, none of the K$^+$-channel blockers (except extra- and intracellularly applied Cs$^+$), high K$^+$, intracellular injections of BAPTA or Mg-ATP, nor TTX had any significant effects on the washout hyperpolarization caused by HS$^-$ (see Figure 24). Lastly, the decrease in neuronal input resistance due to applications of higher concentrations

Fig. 23 Summary of the effects of K^+-channel blockers, TTX, and intracellular injections of Mg-ATP or BAPTA on (A) the initial hyperpolarization, and (B) changed input resistance caused by application of HS^- (200 μM) on CA1 pyramidal cells *in vitro*. All HS^--mediated effects in the presence of the extracellularly-applied drugs (shaded bars) were compared to the controls obtained from the same concentration of HS^- applied to the same neurons. When HS^- was applied to neurons in which Cs^+, Mg-ATP, and BAPTA were injected intracellularly (unshaded bars), the HS^--mediated effects were compared to the responses obtained as a result of application of the same concentration of HS^- to neurons impaled with electrodes filled with KAc. (All values are means ± SEM; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.)


A
Membrane hyperpolarization (% Control)
150
50
-50
-150
-250
-350
*
**
*
***
B
Change in input resistance (% Control)
150
125
100
75
50
25
0
-25
***
*
TTX (1 µM)
TTX (1 µM) + 4-AP (1 mM)
TTX (1 µM) + TEA (50 mM)
Cs⁺ (2 mM)
Cs⁺ (2 M)
Mg ATP (5 mM)
BAPTA (50 mM)

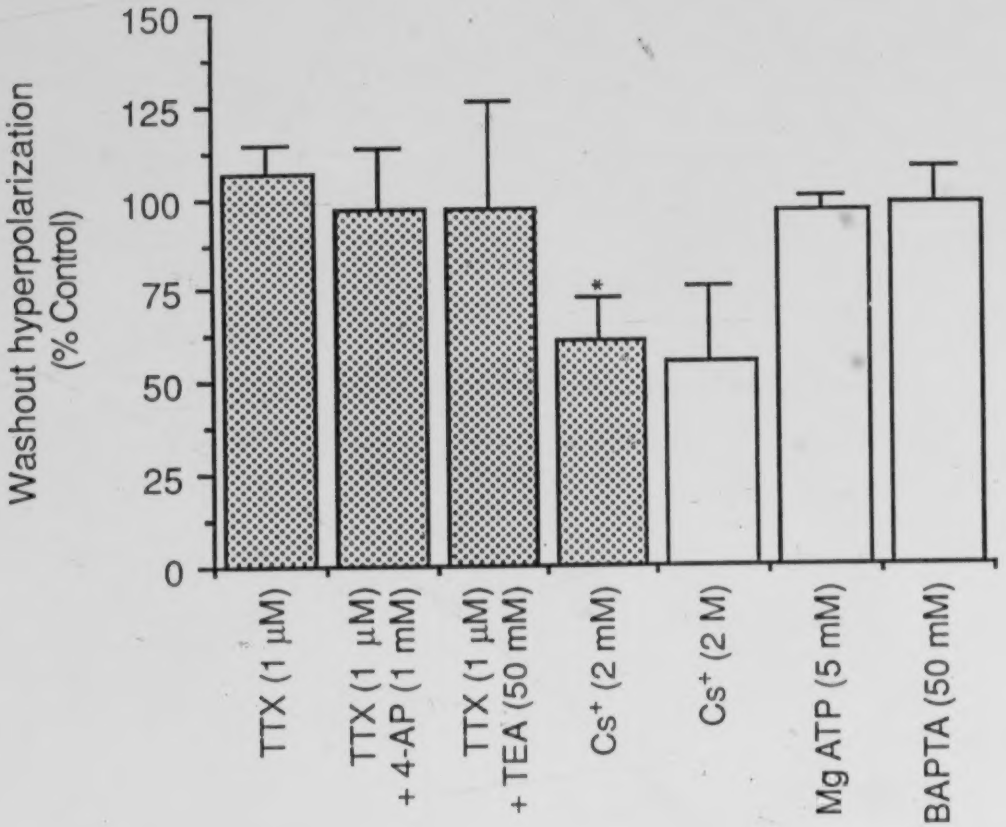


Fig. 24 Summary of the effects of K^+-channel blockers, TTX, and intracellular injections of Mg-ATP or BAPTA on the washout hyperpolarization caused by HS^- (200 μM) on CA1 cells *in vitro*. (All other details as described in Fig. 23.)

of NaHS, often began to recover before the maximum washout hyperpolarization was observed after the wash, suggesting that, unlike the initial hyperpolarization, an increase in membrane conductance was not responsible for the washout hyperpolarization. Together, these results suggested that the underlying mechanisms responsible for the washout hyperpolarization were not identical to those involved in the initial hyperpolarization and that this effect was probably not mediated by activation of a potassium conductance.

To examine the possibility that the HS^--induced washout hyperpolarization was related to the activation of a Na^+-K^+ ATPase, I applied the ouabain-like agent, strophanthidin, which prevents the activation of this membrane pump (Yoda and Hokin, 1970). Extracellular application of 3 μM (n=1), 10 μM (n=1) or 30 μM (n=2) strophanthidin led to a depolarization of the CA1 pyramidal neurons (e.g. 6-8 mV, n=3) which often led to spontaneous action potential activity. Furthermore, although changes in neuronal input resistance were insignificant (e.g. -2.8 ± 4.4 %), strophanthidin caused significant attenuations in EPSP amplitude (e.g. about 75-90 % of controls). However, in the presence of the Na^+-K^+ ATPase blocker, applications of 200 μM HS^- resulted in neither an initial hyperpolarization, nor a washout hyperpolarization (Figure 25). Instead of the initial hyperpolarization (that was seen during HS^- applications in the same neurons (controls)), in the presence of these concentrations of strophanthidin, HS^- caused a depolarization. No changes in membrane potential were observed after wash. However, only partial recovery (e.g. membrane potential and EPSP amplitude) was

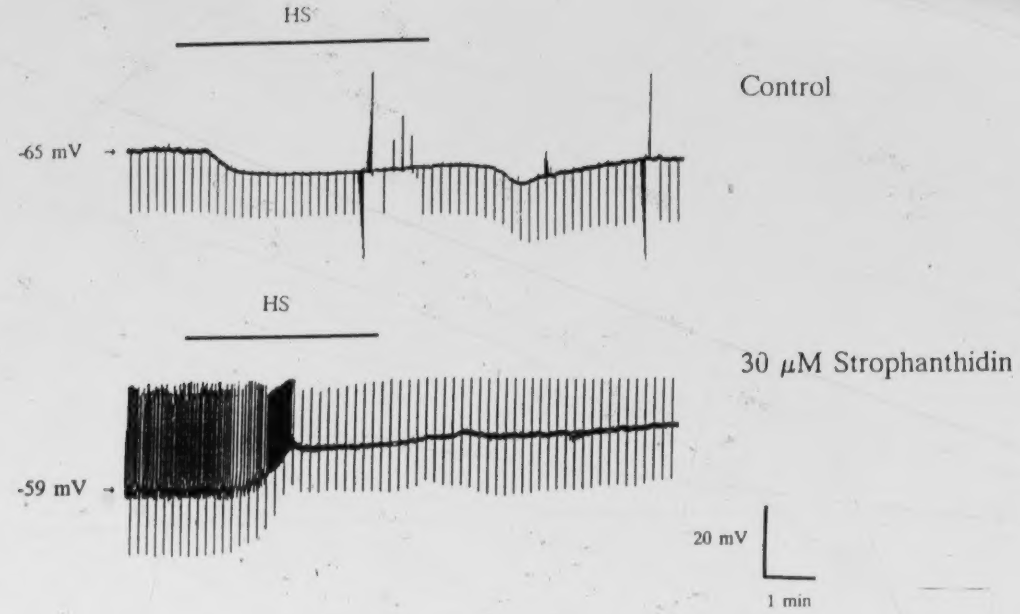


Fig. 25 Effects of strophanthidin on HS⁻-mediated washout hyperpolarization. Chart records of membrane potential of a CA1 cell, downward deflections indicate passage of hyperpolarizing current through the electrode. Bath application of strophanthidin (30 μM) alone led to depolarization of the cells, while input resistance was unchanged. Furthermore, in the presence of strophanthidin, during application of HS⁻ (200 μM), a depolarization (as opposed to a hyperpolarization seen in controls) occurred; in addition, no washout hyperpolarization was observed in these experiments.

obtained even 1 hour after the experiments. Application of lower concentrations of strophanthidin (3 μM) in 1 cell did not suppress the initial hyperpolarization seen during 200 μM HS^-application (e.g. 112.5 % of controls), in addition, the HS^--induced washout hyperpolarization was only decreased by about 16.7 %. Ethanol (50 mM; controls) did not have any effects on the cells when applied alone nor did it lead to any changes in the HS^--mediated effects.

4. Effects of NaHS on synaptic transmission

a. Blockade of evoked synaptic transmission

To investigate whether or not some of the HS^--induced effects were mediated by synaptic release of endogenous neurotransmitters, evoked synaptic transmission was blocked during intracellular recording. This was done using 2 different experimental protocols. In some experiments, the extracellular Ca^{2+} concentration in the perfusion medium was reduced to 0.5 mM $CaCl_2$, and Mg^{2+} was raised to 3.5 mM by addition of $MgCl_2$ to ensure adequate divalent cation concentrations in the ACSF and to maintain cell membrane stability. (Concentrations of all other constituents of the ACSF were identical to those mentioned previously; Colmers et al., 1987.) In other experiments, 1 μM TTX was applied extracellularly to block voltage-dependent Na^+ channels.

In the 3 cells in which low Ca^{2+} (0.5 mM), high Mg^{2+} (3.5 mM) containing ACSF was applied, NaHS (160 μM) caused a direct hyperpolarization, however, the amplitude of this initial hyperpolarization was significantly lower (e.g. to 62.6 ± 11.2 %, n=5; $p< 0.05$), while the decrease in input resistance was unchanged (86.6 ± 19.5 % of controls; $p > 0.5$), compared to the effects seen in the same neurons under normal conditions (Figure 26). The HS^--induced washout hyperpolarizations were also reduced in the presence of low Ca^{2+}, high Mg^{2+} containing media. However, in one cell, application of ACSF containing 0 mM Ca^{2+} (and 4.0 mM Mg^{2+}) did not result in any reduction in the HS^--mediated hyperpolarization (or changes in input resistance). Interestingly, in all cells, a large depolarization (e.g. about 40-50 mV) was observed immediately following the initial hyperpolarization, but the membrane potential recovered completely within 10 minutes.

Application of TTX extracellularly did not reduce the HS^--induced initial hyperpolarization (116.6 ± 15.0 %; n=3) and its associated change in input resistance (112.7 ± 13.8 %) from that of control (see Figure 16 and 23A and 23B). The HS^--mediated washout hyperpolarization was also not affected (106.0 ± 7.9 %; see Figures 16 and 24).

b. Iontophoretic applications of glutamate

The data above suggest that some of the HS^--induced effects such as membrane hyperpolarization and changes in input resistance were not due to action

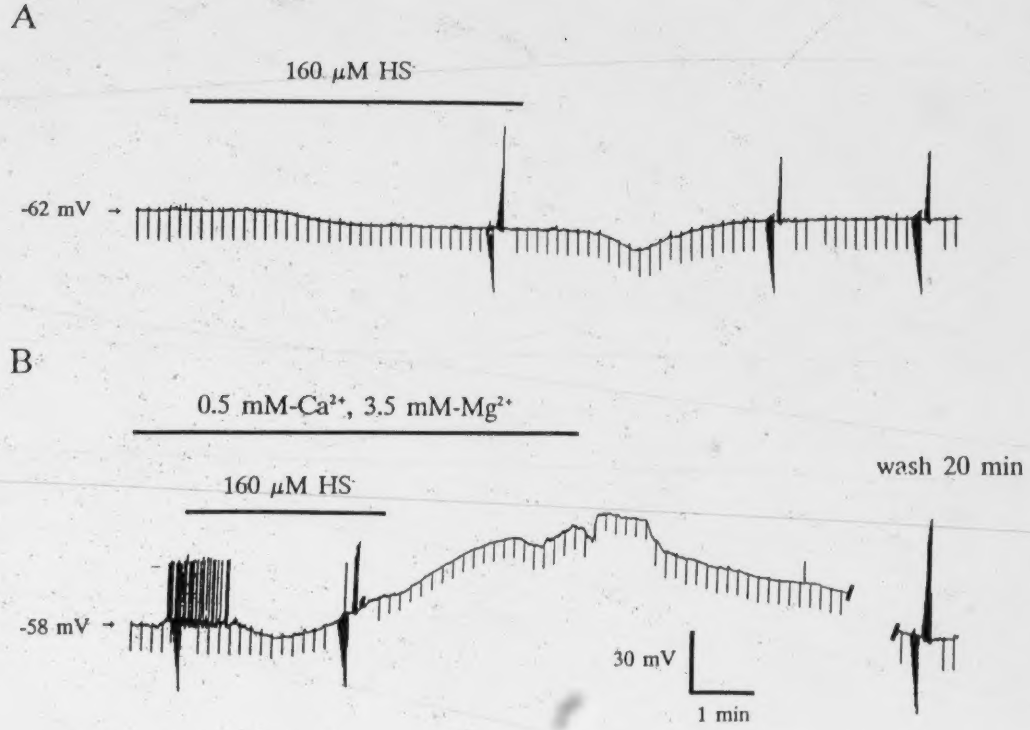


Fig. 26 Effects of low Ca^{2+}, high Mg^{2+} on membrane hyperpolarization and change in input resistance caused by HS^-. Chart records of membrane potential of CA1 neuron of rat hippocampus; downward deflections indicate passage of hyperpolarizing current pulses through the electrode. A, in control medium. HS^- (160 μM) causes a membrane hyperpolarization and decreased input resistance. B, in medium containing low Ca^{2+} (0.5 mM), and high Mg^{2+} (3.5 mM; the cell depolarizes about 3 mV). Application of HS^- (160 μM) causes a smaller hyperpolarization as compared to control (A), however, the HS^--induced decrease is similar to that of control. Furthermore, a depolarization follows the hyperpolarization seen during HS^- application, but the membrane potential recovers after 20 min wash with normal ACSF. Note the decrease in the HS^--induced washout hyperpolarization in the presence of low Ca^{2+}, high Mg^{2+} containing media.

potential mediated changes in transmitter release. However, the location of the direct effects of HS^- on evoked synaptic transmission was not addressed. HS^- might, for example, be acting locally to decrease the membrane resistance of the dendrites or the dendritic spines onto which the terminals of the *stratum radiatum* synapse. This could result in a decreased effect of a given synaptic input to the dendrites or dendritic spines. To investigate this possibility, iontophoretic pulses of glutamate, a putative excitatory transmitter in the Schaffer collateral inputs to CA1 (Wierasko, 1983) were applied to the apical dendrites of impaled CA1 cells near the location of *stratum radiatum*-CA1 synapses. Results from one experiment are shown in Figure 27. Figure 27 (1B) shows the effect of 200 μM HS^- on the orthodromically evoked EPSP in a CA1 neuron, while Figure 27 (2B) shows the averaged response of the neuron to 3 successive iontophoretic pulses of glutamate. During these experiments, the HS^--mediated decrease of the amplitude of the evoked EPSP (85.8 ± 2.2 %, n=4) was significantly greater than the decrease of the responses to the glutamate pulses (51.8 ± 10.8 %, n=4; $p < 0.05$).

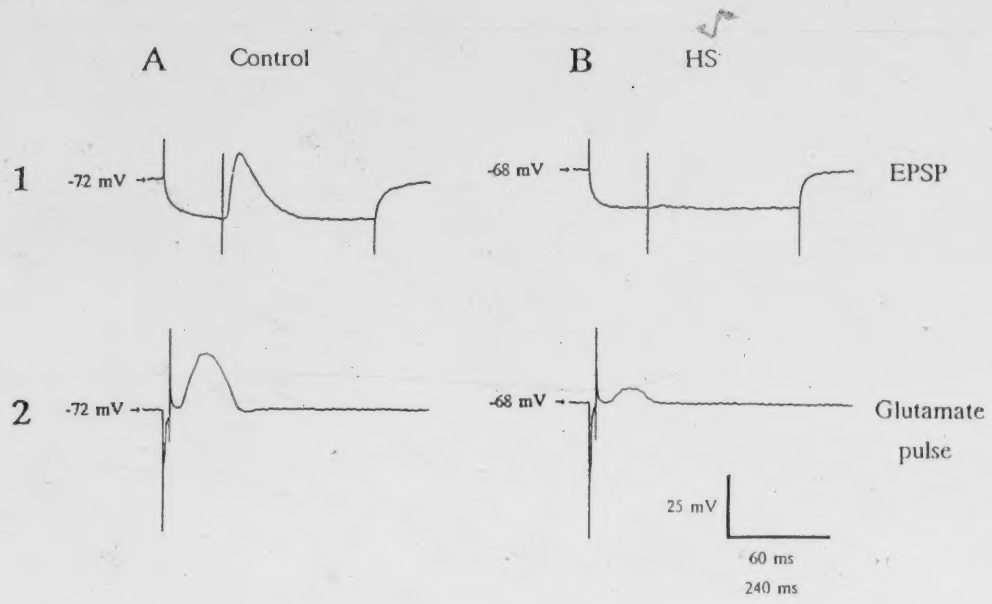


Fig. 27 HS$^-$-mediated decrease of the amplitude of the intracellularly recorded EPSP was greater than the decrease of the responses to glutamate pulses applied iontophoretically to the dendrites of a CA1 pyramidal neuron.

1. EPSP evoked, during a hyperpolarizing current pulse passed via the bridge circuit, by stimulation of *stratum radiatum* 40 ms after start of hyperpolarizing pulse. Each trace is the average of 3 stimuli. 1A, in control medium. 1B, in the presence of 200 μM HS$^-$. Note that the EPSP amplitude is significantly attenuated by HS$^-$ application.

2. Voltage response of a neuron to a 20 ms, 90 nA pulse of glutamate applied to its dendrites. Each trace is the average of 3 pulses. 2A, in control medium. 2B, in the presence of 200 μM HS$^-$. Note that the HS$^-$-induced decrease in the response to the glutamate pulses is smaller than that for the for the EPSP amplitude. Different time scales for 1 (upper value) and 2 (lower value).

CHAPTER IV

DISCUSSION

HS^- rapidly elicits profound, rapidly reversible effects on membrane potential, resistance, and excitability in hippocampal area CA1 neurons of the rat. Many of these effects are elicited at concentrations found in rat brains following i.p. injections of NaHS at or below the LD_{50}, and may therefore be relevant to the toxicology of H_2S. In addition, after application of high concentrations of HS^- (> LD_{99}), a further hyperpolarization was observed subsequent to washout. The latter action may not be significant for the toxicological effects seen at sublethal concentrations of H_2S, but may play a role in the effects seen at universally lethal exposure levels.

The changes in membrane potential and input resistance reversed rapidly and completely upon washout. This concurs with results obtained from experiments *in vivo*; for example, when single cells were treated with iontophoretically applied HS^- several times, to the point of total inhibition of firing, complete and rapid recovery still occurred (see Results I-B). Furthermore, these results are in agreement with observations in some cases of accidental H_2S poisoning, because many victims who have been exposed to high concentrations of H_2S, and experience respiratory arrest, recover quickly when removed to fresh air and mechanically respired (Milby, 1962).

A. Mechanism of Initial Hyperpolarization and Input Resistance Decrease

1. Nature of ionic conductance altered by HS^-

Application of NaHS caused a hyperpolarization and a decrease in neuronal input resistance in CA1 pyramidal cells. The reversal potential for this conductance change, estimated from current- and voltage-clamp data, was more negative than -95 mV. Although the estimated value of the reversal potential was more positive than the potassium equilibrium potential (E_K) for these cells ($E_K \simeq -105$ mV), this was not surprising since the limitations of the techniques used would likely contribute to an underestimated value of the reversal potential. However, the activation of Na^+ and/or Ca^{2+} conductances during HS^- application could also contribute to a lower value of the reversal potential for the HS^--induced conductance change. When the reversal potential was shifted in a depolarizing direction by elevating extracellular potassium concentrations (Kuffler et al., 1984; Segal and Barker, 1984), the hyperpolarization induced by HS^- was significantly reduced. Furthermore, results from experiments in which CA1 cells were impaled with electrodes filled with KCl (2 M) indicated that the reversal potential for the HS^--mediated conductance change was insensitive to changes in intracellular Cl^- concentrations. Together, these results suggested that the hyperpolarization and its associated decrease in input resistance caused by $HS^-$was most likely as a result of an increased conductance to potassium in CA1 pyramidal neurons. This HS^--

mediated effect was probably not mediated presynaptically as it was not affected when synaptic transmission was blocked by application of extracellular TTX. However, blockade of synaptic transmission by perfusion of low Ca^{2+}, high Mg^{2+} ACSF did lead to a reduction in the hyperpolarization, but not in the change in input resistance caused by HS^-, in 2 out of 3 neurons. In the third neuron, the HS^--induced effects were not affected in the presence of 0 mM Ca^{2+}, 4.0 mM Mg^{2+}.

Further evidence that supported the hypothesis that an increased K^+-conductance accounted for the effects seen during HS^- application included results obtained from experiments involving applications intracellular Cs^+ (2 M), which has been reported to block several K^+-currents (Colmers et al., 1982; Johnston et al., 1980; Puil and Werman, 1981). In these experiments, when CA1 neurons were injected with Cs^+, no initial HS^--mediated hyperpolarization was observed; instead, application of HS^- caused a strong depolarization which was associated with a large decrease in input resistance of these cells, indicating that the potassium-dependent hyperpolarization masks an additional, potassium-independent conductance change.

Some cells were insensitive to the hyperpolarization and decreased input resistance caused by application of 200 μM HS^- (n=6). These cells tended to have more negative resting potentials and lower input resistances, thus it was possible that no further hyperpolarization or drop in input resistance occurred in response to HS^- application because many of their potassium conductances were already activated.

2. *Nature of HS^--activated K^+-conductance*

Since several types of K^+-channels have been discovered in hippocampal neurons (Rudy, 1988; Segal and Barker, 1984; Halliwell and Adams et al., 1982a), several K^+-channel blockers were used to determine which type(s) of K^+-conductances were contributing to the HS^--induced hyperpolarizations.

a. Voltage dependent K^+-channels

Since the HS^--mediated effects were insensitive to high concentrations of 4-AP, it is not likely that the A current (I_A) was involved (Rudy, 1988; Segal and Barker, 1984). Furthermore, this hypothesis was also supported by the fact that I_A activates (e.g. peaks) within 3-5 ms and inactivates rapidly (< 30 ms; Segal and Barker, 1984) in response to changes in membrane potential.

There was some evidence to suggest that the delayed rectifier, I_K, was probably not contributing to the effects caused by HS^-. For example, according to Segal and Barker (1984), a full depression of I_K was observed in CA1 pyramidal cells after extracellular application of 20-25 mM TEA, whereas TEA had no significant effects on the hyperpolarization and the decrease in input resistance caused by HS^-. Moreover, even the high dose (50 mM) of TEA applied in these studies only led to about a 50 % reduction in the HS^--mediated hyperpolarization and its associated change in input resistance. Application of extracellular Cs^+ also blocks I_K (Hille, 1984; Rudy, 1988), however, in these experiments Cs^+ suppressed the hyperpolarization but not the change in input resistance caused by HS^-.

It is difficult to conclude whether or not the voltage-dependent potassium M current (I_M) contributes to the initial HS^--mediated effects. Although I_M has been reported to be deactivated at membrane potentials below -62 mV in hippocampal neurons (Halliwell and Adams, 1982), it is still possible that the activation of I_M could account for the effects observed during HS^- application. For example, there is some evidence that suggests that somatostatin, a peptide neurotransmitter substance which is found throughout the brain (Elde and Hokfelt, 1981), activates the M current in pyramidal neurons of the hippocampus (Moore et al., 1988; Watson and Pittman, 1988). Interestingly, the conductance activated by somatostatin is not sensitive to extracellular TEA (10 mM), 4-AP (100-500 μM), and extracellular Cs^+ (1 mM; Pittman and Watson, 1987; Watson and Pittman, 1987). This is pharmacologically similar to the conductance change induced by sulfide. Thus, HS^- may also activate I_M (either directly or via activation of the somatostatin receptor), and thus account for the hyperpolarization and decreased input resistance seen during HS^- application.

b. Inwardly rectifying K^+-conductances

It was difficult to conclude whether or not an inwardly-rectifying K^+-conductance was contributing to the HS^--induced effects. The best understood inward rectifier, the Q current (I_Q), has been shown to be significantly reduced by applications of extracellular Cs^+ and Ba^{2+}, or Na^+-free medium in hippocampal neurons (Halliwell and Adams, 1982; Segal and Barker, 1984; Rudy, 1988). The evidence is conflicting. Application of extracellular Cs^+ (2 mM) did not lead to a reduction in the HS^--mediated change in input resistance which suggests that I_O was not involved, but on the other hand, the initial hyperpolarization caused by HS^- was significantly reduced, suggesting a role for I_O.

c. ATP-sensitive K^+-channels

As mentioned above, it has been suggested that H_2S may exert its toxic effects via inhibition of mitochondrial oxidative phosphorylation (Beauchamp et.al., 1984; Finklea, 1977; Smith, 1979), similar to hydrogen cyanide (Smith et al., 1977). This would lead to a depletion of intracellular ATP, and possibly the activation of an ATP-sensitive K^+-conductance (gK_{ATP}). The evidence suggests that the activation of this conductance is not likely to account for the HS^--induced effects seen in CA1 cells. Intracellular injection of 5 mM Mg^{2+}-ATP (in 2 M KAc), which should block these channels (Cook and Hales, 1984), did not alter any of the HS^--mediated effects. Furthermore, although ATP-sensitive K^+ channels (K_{ATP}) have been found in cardiac muscle (Noma, 1983; Kakei and Noma, 1984), in pancreatic ß cells (Cook and Hales, 1984), and recently in the presynaptic terminals (e.g. the mossy fibres) that synapse onto CA3 pyramidal cells of the rat hippocampus (Melander et al., 1986; Mourre et al., 1989), a K_{ATP}-sensitive conductance has not been observed in CA1 pyramidal cells of the hippocampus. Direct evidence must be obtained to demonstrate the presence of this conductance in these cells, and if it is present, the pharmacological properties of this conductance in intact CA1 pyramidal cells must be determined. Until results from studies suggest that these channels are present in CA1 cells, one can assume that gK_{ATP} does not play a major role in the initial HS^--induced hyperpolarization in these cells.

d. Leak conductances

It is possible that the initial hyperpolarization and decrease in input resistance seen during HS^- application may be due to an increase in a leakage current (Adams et al., 1982b). Using whole cell recordings of isolated frog sympathetic neurons, Jones (1989) reports that the resting potential of these neurons depends mainly on a relatively voltage-insensitive leakage current. The reversal potential for this current is about -65 mV, and shifts in a depolarizing direction with increasing extracellular potassium concentrations, and thus, is a reasonably selective K^+-current (Jones, 1989). Furthermore, this current is distinct from I_Q, I_K, gK_{Ca2+}, or I_M as it is not blocked by any antagonists to these conductances. The fact that there are no specific blockers for I_A in these cells (Adams et al., 1982b) made it difficult to test whether or not it is I_A. Hence, given some of the pharmacological similarities between this leakage current and the conductance which is activated during HS^- application, it is possible that the leakage current may contribute to the initial hyperpolarization and the decreased input resistance caused by HS^-.

e. Ca^{2+}-sensitive K^+-conductance

Based on our results, it was difficult to conclude whether or not gK_{Ca2+} was responsible for the initial hyperpolarization and decreased input resistance caused by HS^-. For example, intracellular injections of the Ca^{2+} chelator, BAPTA, which supposedly blocked gK_{Ca2+} in these cells (Lancaster and Nicoll, 1987; Storm, 1987) did lead to some reduction, but not a complete blockade, in the HS^--induced hyperpolarization and decreased input resistance as compared to controls. There was evidence to suggest that some BAPTA was present in these cells, as seen by action potential spike broadening (probably due to inhibition of the fast Ca^{2+}-activated K^+-current by BAPTA; Lancaster and Nicoll, 1987; Storm, 1987), as well as some decrease in the slow afterhyperpolarization (AHP) which was also due to the BAPTA-induced inhibition of gK_{Ca2+} (Lancaster and Nicoll, 1987; Storm, 1987). However, these BAPTA-mediated effects were not as obvious as the effects reported by others when CA1 cells were impaled with electrodes filled with BAPTA (in KAc or KCl). For example, Storm (1987) showed that application of intracellular BAPTA led to about a 50 % increase in action potential duration (spike width measured at about one-third of full amplitude as was done in our experiments) as well as a complete blockade of the Ca^{2+}-dependent slow AHP. There are several possible explanations for the smaller effects observed by intracellularly-applied BAPTA in our experiments. For example, in hippocampal neurons impaled with electrodes filled with concentrations (e.g. 50 to 200 mM) of BAPTA in KCl or KAc, the fast AHP has been reported to be significantly depressed (Lancaster and Nicoll, 1987; Storm, 1987), but in our experiments, successful intracellular recordings could only be obtained from cells impaled with electrodes filled with 50 mM BAPTA, one of the lowest concentrations reported to block the fast AHP. Furthermore, since the electrodes used in our experiments had somewhat higher resistances (e.g. 70-100 MΩ) as compared to those used by others (e.g. 20-80 MΩ; Lancaster and Nicoll, 1987; Storm, 1987), the amount of BAPTA that diffused into the cells in our experiments would probably be lower than the amounts of BAPTA found intracellularly in their experiments even though all cells may have been impaled with electrodes filled with the same concentrations of BAPTA. This would have led to a less effective blockade of gK_{Ca2+} in our experiments. Lastly, applications of BAPTA may have been ineffective in completely blocking the HS^-- effects because the K^+-channels involved may be inaccessibly located or their sensitivity to Ca^{2+} is very high (Adams et al., 1985; Byerly et al., 1984, 1986; Kramer and Zucker, 1985; Krnjević and Leblond, 1989). Therefore, some or all of these factors may explain the lack of complete protection from the effects caused by HS^- by intracellular injections of BAPTA, and hence make it difficult to prove conclusively whether or not gK_{Ca2+} is *responsible* for the effects seen during HS^- application although it appears to *contribute*.

3. HS^--mediated depolarizations observed during some experimental conditions

Under certain conditions of altered extra- or intracellular environments, the application of HS^- resulted in a reversible depolarization which was associated with a decrease in input resistance. This was observed when K^+-conductances were blocked by intracellular Cs^+, or when extracellular K^+ was elevated or when extracellular Ca^{2+} was lowered. While these observations were contradictory to the HS^--mediated effects seen under most conditions, they were often marked and consistent under these conditions.

When intracellular injections of Cs^+ were applied to CA1 neurons, no initial, HS^--mediated hyperpolarization was observed; instead application of HS^- caused a depolarization (e.g. 20-30 mV) which was associated with a large decrease in input resistance of these cells, indicating that the potassium-dependent hyperpolarization masks an additional, potassium-independent conductance change. Interestingly, the rate of onset of the depolarizations seen in these experiments was similar to the rate of onset of the HS^--induced hyperpolarizations seen in experiments in which cells were impaled with KAc-filled microelectrodes. It is likely that this HS^--induced, potassium-independent conductance increase was probably due to the activation of either a Ca^{2+}- or Na^+-current, since the increased inward conductance of either of these ions would explain the HS^--mediated depolarization seen in the presence of intracellular Cs^+. It is therefore possible that HS^- can activate a Ca^{2+} conductance, leading to an influx of extracellular Ca^{2+}, and possibly the release of Ca^{2+} from internal stores. This would increase the intracellular Ca^{2+} concentration, and thus activate a Ca^{2+}-activated K^+-conductance. Because this K^+-conductance was blocked in the presence of intracellular Cs^+, the HS^--induced hyperpolarization normally seen in CA1 cells impaled with electrodes filled with KAc was not observed, and the influx of Ca^{2+} unmasked as a depolarization. However, there were some results that conflicted with this hypothesis. For example, in some experiments, during applications of intracellular Cs^+ in CA1 cells (n=2), the amplitude of the HS^--induced depolarization was not affected in the presence of low Ca^{2+}, high Mg^{2+}-containing media. On the other hand, the HS^--induced depolarizations seen in these experiments could have been as a result of an influx of extracellular Na^+, which could lead to the activation of a Na^+-dependent K^+-conductance (gK_{Na}; Schwindt et al., 1988; 1989), and the HS^--mediated hyperpolarization seen in cells impaled with electrodes filled with KAc. This Na^+-dependent K^+-current has been shown to underlie the slow AHP seen in neocortical neurons and was responsible for the reduction of excitability observed following stimulation of these neurons.

During HS^- applications in high-potassium-containing media, a depolarization (e.g. 20 mV) was observed following the initial hyperpolarization. The application of high concentrations of extracellular K^+ (which also shifted the reversal potential in a depolarizing direction), coupled with the activation of a K^+-conductance by sulfide, changed the K^+-concentration gradient across the membrane. This alteration in the concentration gradient for K^+ would have led to an influx of some K^+ into the cell, and thus, may have accounted for the sulfide-induced depolarization seen in the CA1 cells recorded under these conditions. Furthermore, the high concentrations of extracellular K^+ present in these experiments may have caused a depolarization of the presynaptic terminals which synapse onto CA1 cells, and thus resulted in an increased transmitter release from these terminals. This would have led to a further depolarization (in addition to the depolarization caused by the altered K^+-concentration gradient) seen in the CA1 cells recorded in these experiments.

In some experiments (n=5), the initial hyperpolarization, but not the change in input resistance, caused by HS^- was reduced in the presence of low Ca^{2+}, high Mg^{2+}-containing ACSF. Furthermore, a depolarization (e.g. up to 40 mV) was observed immediately following the initial HS^--induced hyperpolarization seen under these conditions. It was likely that gK_{Ca2+} was almost completely blocked in these experiments because the extracellular concentration of Ca^{2+} was very low (Hille, 1984). Thus, if HS^- was activating gK_{Ca2+}, this could explain the smaller hyperpolarization (as compared to controls in the presence of normal ACSF) seen during HS^- applications in these experiments. The HS^--mediated depolarization seen in the presence of low Ca^{2+}, high Mg^{2+} could be attributed to the activation of a Na^+-current (as the input resistance decreased during the depolarization) or perhaps, due to the inhibition of the Na^+-K^+ ATPase during the HS^- application.

4. Similarities between HS^-- and anoxic-induced effects

Many studies have examined the effects of anoxia on CA1 pyramidal neurons using the *in vitro* hippocampal slice technique (Hansen et al., 1982; Fujiwara et al., 1987; Leblond and Krnjević, 1989; Misgeld and Frotscher, 1982). Interestingly, the hyperpolarization and decrease in input resistance caused by HS^- in our experiments were similar to the actions induced by anoxic conditions in those studies. In addition, the anoxia-induced hyperpolarization seen in CA1 cells was reduced by increasing the concentration of extracellular potassium and there was evidence to suggest that an increased membrane potassium conductance may underlie these effects (Fujiwara et al., 1987; Hansen et al.,1982; Leblond and Krnjević, 1989). However, the precise potassium conductance(s) affected by anoxia have not yet been identified.

Fujiwara et al. (1987) hypothesized that the effects observed in the CA1 cells under anoxic conditions were due to activation of a voltage-independent K^+-conductance (e.g. gK_{ATP}), but this hypothesis was based on indirect evidence. However, Leblond and Krnjević (1989) found that when CA1 neurons were impaled with microelectrodes filled with 100-200 mM ATP, they observed only small reductions in the changes in input resistance in response to anoxia, hence conflicting with the hypothesis that gK_{ATP} is likely to contribute to the anoxic-induced initial hyperpolarization and decreased input resistance seen in CA1 pyramidal cells. This concurred with the results obtained in our experiments in which application of 5 mM Mg^{2+}-ATP, intracellularly did not alter any of the HS^--mediated effects.

On the other hand, Krnjević and Xu (1989b, 1989c) have some evidence to suggest that a Ca^{2+}-sensitive potassium-conductance may account for the initial effects seen during anoxia as extracellular applications of dantrolene, which blocks the release of Ca^{2+} from internal stores (Desmedt and Hainant, 1977) suppressed the effects observed in CA1 neurons during anoxia. Thus, they hypothesized that anoxia activates gK_{Ca2+} by releasing Ca^{2+} from internal stores (Krnjević and Xu, 1989b; 1989c). Furthermore, hydrogen cyanide, which is thought to exert its effects by a similar mechanism to that of H_2S (Beauchamp et al., 1984; Smith , 1979), has also been shown to release calcium from intracellular stores, thereby increasing gK_{Ca2+} in carotid Type I chemosensory cells (Biscoe and Duchen, 1989; Biscoe et al., 1989).

B. Washout Hyperpolarization

Higher concentrations of HS^- caused a further hyperpolarization shortly after washout was initiated. The amplitude of the washout hyperpolarization was dependent on HS^- concentration, but was independent of the duration of HS^- application. Unlike the initial hyperpolarization observed during HS^- application, the washout hyperpolarization was not associated with a increased membrane conductance because the neuronal input resistance began to recover before the maximum washout hyperpolarization was observed after the wash. This hyperpolarization was not decreased by application of high-potassium-containing media, any of the K^+ channel blockers (except for intra- and extracellularly applied Cs^+; see below), or intracellular injections of Mg-ATP or BAPTA. Together, these results suggested that the underlying mechanisms responsible for the HS^--induced washout hyperpolarization were different from those involved in the initial hyperpolarization seen during HS^- application, and that this effect was not due to an increased potassium conductance.

Furthermore, since the HS^--mediated washout hyperpolarization was blocked by the Na^+-K^+ ATPase inhibitor, strophanthidin (Yoda and Hokin, 1970), it was likely that the washout hyperpolarization may have been due to reactivation of the electrogenic Na^+-K^+ ATPase during the wash after application of HS^-. Since the washout effect was often greater in magnitude than the initial hyperpolarization, one must conclude that the Na^+-K^+ ATPase was blocked by HS^-, and that this blockade was easily reversible by removal by of HS^-. Also, if HS^- had activated an outward K^+ current, this would have led to a change in the K^+-concentration gradient across the membrane which may have existed for some time after wash. The changed K^+ gradient would then activate the Na^+-K^+ ATPase (once the inhibition caused by HS^- was removed) to restore the K^+ concentration gradient (Rang and Ritchie, 1969).

These results concurred with other studies which reported that a late hyperpolarization was observed in anoxic CA1 pyramidal cells subsequent to reperfusion with oxygenated medium (Hansen et al., 1982; Fujiwara et al., 1987; Krnjević and Leblond, 1989a; Leblond and Krnjević, 1989) as this could be selectively blocked with ouabain and strophanthidin (Fujiwara et al., 1987; Leblond and Krnjević, 1989). They also found that this washout hyperpolarization was blocked by the removal of potassium ions (Fujiwara et al., 1987) perhaps, something we could have done in our experiments. Furthermore, higher concentrations of HS^- than those used in this study were shown to potentiate the activity of a Na^+-K^+ ATPase in bullfrog sympathetic ganglia, but only following removal of HS^-, and not during exposure to HS^- (Kombian et al., 1988b).

However, instead of an initial hyperpolarization as was seen during HS^- applications in the same neurons (controls), in the presence of these concentrations of strophanthidin a HS^--induced depolarization was observed. Thus, this conflicted with our hypothesis that the underlying mechanisms responsible for the initial hyperpolarization and washout hyperpolarization mediated by HS^- were different. Interestingly, Fujiwara et al. (1987) reported that instead of an anoxic-induced initial hyperpolarization which was normally observed in CA1 cells, a depolarization was seen when these cells were exposed to anoxic conditions in the presence of ouabain (7 μM).

Intra- and extracellular application of Cs^+ leads to a reduction in the washout hyperpolarization caused by HS^-. Although further experiments are required to determine the underlying mechanisms for these observed effects, it is possible that Cs^+ could decrease the efficiency of the Na^+-K^+ pump by competing with either Na^+ (e.g. intracellularly) or the K^+ (e.g. extracellularly) for the target sites on the pump.

Interestingly, intracellular injections of BAPTA and extracellular application of TEA (50 mM) led to a reduction in the initial hyperpolarization and decreased input resistance caused by HS^-, but the HS^--induced washout hyperpolarization (measured from the resting membrane potential preceding NaHS application) was not affected. Hence, the maximum change in membrane potential observed after wash (measured from the membrane potential found immediately after the termination of NaHS application) was much larger in these experiments (e.g. 154.3 ± 13.1 %, and 174.1 ± 64.0 %, respectively) than those observed in controls. Because intracellular BAPTA and extracellular TEA suppressed the HS^--induced changes in input resistance, a greater voltage change (e.g. washout hyperpolarization) may have been caused by the (presumably constant) pump current generated across the membrane when the electrogenic Na^+-K^+ exchanger was reactivated after initiation of washout (Rang and Ritchie, 1969).

C. Effects on Synaptic Transmission

The results indicate that HS^- (60 μM) alters the relationship (qualitatively, by about 50 %) between the extracellularly recorded field EPSP amplitude and PS amplitude. This suggests that HS^- may alter the cable properties of CA1 cells, thus decreasing the ability of these cells to generate action potentials in response to excitatory synaptic input. Furthermore, higher concentrations of HS^- (200 μM) cause about a [illegible] % reduction in CA1 neurons' responses to iontophoretic applications of glutamate to their dendrites while the EPSP amplitude, evoked by *stratum radiatum* stimulation, is decreased by about 85 % in the same cells. Also, the decrease in input resistance of the soma of CA1 pyramidal cells (about 40 %), in response to application of 200 μM HS^-, is about the same as the changes seen in the CA1 cells' responses to iontophoretic pulses of glutamate.

Since iontophoretic application of glutamate to the dendrites of CA1 cells leads to a local effect, the results obtained from such experiments suggest that HS^- probably reduced the input resistance of the dendrites or the dendritic spines of the CA1 pyramidal cells. Interestingly, the reduction in the CA1 neurons' responses to glutamate pulses in these experiments was similar to the decrease in input resistance of the soma caused by HS^- application. Furthermore, since application of lower concentrations of HS^- (60 μM) led to a shift in the PS-field EPSP relationship (see Figure 12), this indicates that HS^- causes a change in electrotonic conduction of excitation from the dendrites to the soma which could be attributed to a HS^--mediated decrease in the input resistance of the dendrites of CA1 cells. So far, only the postsynaptic effects of sulfide have been discussed, however, there is some evidence to suggest that sulfide could be acting presynaptically. Although the initial hyperpolarization and decrease in input resistance caused by HS^- was reduced by intracellular injections of Cs^+ and BAPTA, the synaptic response was still sharply attenuated by application of HS^-. Furthermore, the HS^--mediated decrease in amplitude of the evoked EPSP was significantly larger than the decrease of the responses to the glutamate pulses. Therefore, HS^- may be acting presynaptically, possibly by increasing the K^+-conductance of the presynaptic terminals. If so, then the shunting effect of this increased conductance could lead to a reduction in the evoked release of transmitter by the presynaptic terminals which synapse onto CA1 cells.

The results indicate that HS^- causes a larger reduction in the orthodromically evoked population spike (PS) in area CA1 than the EPSP recorded from the pyramidal cells in this area. The PS is a reflection of the extracellular field potential generated by the simultaneous action potential discharge of a large number of cells (Andersen et al., 1971a). The extracellularly recorded field EPSP represents the response of the dendrites of many CA1 pyramidal cells to excitatory synaptic input evoked by *stratum radiatum* stimulation and is related linearly to the intracellularly recorded EPSP (Andersen et al., 1978). A sigmoidal relationship exists between the amplitudes of the PS and the field EPSP, with a relatively steep slope occurring in the middle portion of the relationship (Andersen et al., 1978). Since HS^- decreases the amount of excitatory synaptic input to the cells, a shift in the relationship between the PS and EPSP into the steep portion of the curve occurs. Hence, any small reduction in the amplitude of the EPSP causes a disproportionate reduction in the number of neurons reaching action potential threshold, which results in a relatively larger reduction in the amplitude of the PS. Thus, the differences of the observed effects of HS^- on the two parameters can be explained by: (1) the reduction in EPSP amplitude, and (2) its effects on the cable properties of the cell (as discussed above).

Our data indicate that HS^- (80 μM) does not alter active membrane properties including action potential amplitude and duration, as well as action potential threshold of the postsynaptic CA1 cell. This concurs with results obtained from experiments in which application of NaHS did not alter TTX-sensitive Na^+-channels in mouse neuroblastoma cells (Warenycia et al., 1989d). However, in some cells, the slow AHP was reduced by HS^-, and this could be attributed to the shunting effect caused by the HS^--mediated increased K^+-conductance.

D. Suggestions For Further Experiments

In order to determine precisely whether or not I_K, I_M, or a leak conductance contributes to the initial hyperpolarization and decreased input resistance seen during HS^- application, it would be interesting to examine the actions of HS^- in the presence of extracellular Ba^{2+} (1 mM) which blocks these conductances (Adams et al., 1982a; Halliwell and Adams, 1982; Hille, 1984; Rudy, 1988). In addition, since I_M is blocked by extracellular application of muscarinic agonists such as muscarine and carbachol (Adams et al., 1982a; Halliwell and Adams, 1982) it would have been interesting to test HS^- in their presence as well. Further experiments involving applications of extracellular Ba^{2+} and Cs^+ are required to determine precisely whether or not I_Q contributes to the initial hyperpolarization and decreased input resistance caused by HS^-.

To test the hypothesis that the HS^--mediated depolarizations seen during applications of intracellular Cs^+ or in the presence of low Ca^{2+}, high Mg^{2+} are due to the activation of inward Na^+-conductances, HS^- should be tested in the presence of a Na^+-free medium. Experiments involving applications of HS^- in the presence of intracellular Cs^+ or low Ca^{2+} should also be performed in the presence of dantrolene (which blocks the release of Ca^{2+} from intracellular stores; Desmedt and Hainant, 1977) or thapsigargin (which inhibits the endoplasmic reticulum Ca^{2+}-ATPase in rat hepatocytes; Thastrup et al., 1990) to investigate whether or not HS^- causes the release of calcium from intracellular stores, and thus, is the mechanism responsible for the depolarization caused by HS^- under these conditions. If the application of dantrolene or thapsigargin blocks the HS^--mediated depolarization seen under these conditions, this would support the hypothesis that HS^- activates $gK_{Ca^{2+}}$.

One can hypothesize that the HS^--mediated depolarization seen under conditions of elevated extracellular K^+ could be due in part to increased transmitter release from the presynaptic terminals which synapse onto CA1 cells. Since there is good evidence that suggests that glutamate is the transmitter released from these terminals (Storm-Mathisen, 1977), application of glutamate receptor antagonists such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Honré et al., 1988), and D-2-amino-5-phosphonovalerate (APV; Collingridge, 1985) would enable us to determine whether or not this hypothesis is correct.

Furthermore, because the effects observed in CA1 cells caused by HS^- are similar to those observed during anoxia, it would be interesting to examine the effects of HS^- on CA3 cells. A recent study (Ben Ari and Lazdunski, 1989) reported that anoxia causes different effects in CA3 cells as compared to those observed in CA1 neurons (Leblond and Krnjević, 1989). If HS^- causes similar effects in CA3 pyramidal cells as those seen during anoxic conditions, this would also support the hypothesis that similar underlying mechanisms may account for the effects caused by HS^- and by anoxia.

Because it has been traditionally thought that HS^- exerts its toxic effects via similar mechanisms to that of hydrogen cyanide (Beauchamp et al., 1984; Smith, 1979), and because it has been demonstrated that cyanide activates gK_{Ca2+} (Biscoe and Duchen, 1989; Biscoe et al., 1989), it would be interesting to examine the effects of cyanide on CA1 pyramidal cells. Since the EC_{50} for the HS^--mediated inhibition of cytochrome aa3 is lower than the EC_{50} for the inhibition of the same enzyme by cyanide (Smith, 1979), concentrations of cyanide will have to be matched accordingly.

E. Summary and Conclusions

The object of this study was to examine the effects on neuronal function of the industrial toxin, H_2S, using the hippocampus as a model system. Initially, *in vivo* electrophysiological recording techniques were performed, and the results from these experiments suggested that iontophoretically applied HS^- reversibly inhibits the activity of spontaneous and glutamate-driven CA1 pyramidal cells of the hippocampus, however, the concentration of HS^- applied cannot be easily determined in this type of experiment. Since the *in vitro* hippocampal slice technique offered numerous advantages over the *in vivo* technique (Dingledine, 1984), electrophysiological recordings of CA1 cells *in vitro* were performed. This allowed us to examine the mechanism of action of H_2S in greater detail, and to determine whether or not toxicologically relevant concentrations of HS^- altered neuronal properties.

The results obtained during the *in vitro* experiments suggest that sublethal concentrations of HS^- may inhibit neuronal activity in the hippocampus reversibly by suppressing synaptic responses and by direct hyperpolarization of postsynaptic neurons. These results suggest that the initial HS^--induced hyperpolarization observed in CA1 neurons is most likely due to an increased potassium-conductance. While the nature of the potassium-conductance(s) affected is still unknown, evidence suggests that a calcium-dependent, potassium-conductance may be involved. Furthermore, HS^- clearly causes a decrease in the input resistance of the soma, and thus possibly also of the dendrites or the dendritic spines of CA1 pyramidal cells. In addition, there may be an underlying inward conductance which is normally masked by the activation of a potassium conductance. As well, HS^- may cause a reduction in the release of excitatory neurotransmitters from presynaptic terminals which synapse onto the dendrites of CA1 neurons.

Applications of high concentrations of HS^- ($> LD_{90}$) causes a further hyperpolarization which is observed subsequent to washout. This latter effect, which is most likely due to the reactivation of the Na^+-K^+ ATPase, may not account for the effects seen at sublethal concentrations of H_2S, but may play a role in the effects observed at universally lethal exposure levels.

The HS^--induced suppression of synaptic input and initial hyperpolarization observed in the hippocampus may be responsible for some of the clinical manifestations such as retrograde amnesia seen in survivors exposed to high concentrations of H_2S (Ahlborg, 1951; Burnett et al., 1977). Should similar actions of HS^- occur in brainstem respiratory networks, the resulting inhibition of the rhythmic neuronal oscillators could lead to failure of central respiratory drive, the acute toxic effect seen upon exposure to H_2S (Beauchamp et al., 1984; Milby, 1962). Finally, because the effects of H_2S and anoxia are so markedly similar, it is likely that there are similar underlying mechanisms involved. Since the concentrations of H_2S can be regulated to induce some of these effects, it may prove to be a useful model for understanding the mechanisms of anoxia.

CHAPTER V

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